A STUDY TO DETERMINE THE FEASIBILITY OF USING PHYSICAL METHODS FOR BIOCHEMICAL ANALYSIS UNDER SPACE FLIGHT CONDITIONS

FINAL REPORT

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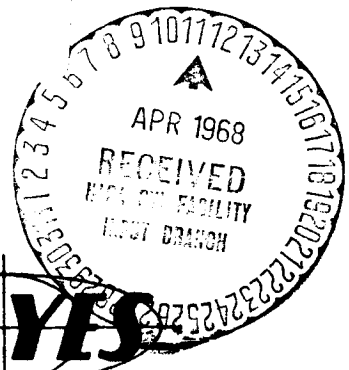
HAYES

INTERNATIONAL CORPORATION

BIRMINGHAM, ALABAMA

SUMMARY

Hayes International Corporation has conducted an eight-month study to determine the feasibility of using physical methods in biochemical analyses under space flight conditions.

The program was initiated with a series of literature searches, both computerized and manual, culminating in a massive search of <u>Chemical Abstracts</u> for the period from 1906 through 1966. The result of the literature searches is a comprehensive survey of analytical techniques and methodology, effectively defining the present state of the art relative to the biochemical constituents included in this study. The review of each constituent is complete in itself, including a merit evaluation of analytical methods with effective figures of merit and an annotated bibliography. This information is presented in summary form in a table. A concerted attempt was made to find or propose new physical methods and techniques of analysis. Three new analytical methods are presented in a separate section.

Based on the findings of this study, a number of recommendations concerning the further development of methodologies are offered.

CONCLUSIONS

As a result of this feasibility study, the following general conclusions have been drawn.

1. At the present time most analytical techniques, conceived and developed in terms of chemical analyses, cannot be readily altered to substantially reduce the amount of wet chemistry. In a few cases it has been suggested that filter paper be impregnated with reagents.

2. Several constituents, notably adrenocorticotrophic hormone, antidiuretic hormone, serotonin, and aldosterone, show little potentiality for effective assay in an orbiting laboratory. Indirect measurement, however, may in time become feasible.

3. The development of new physical methods, such as microcalorimetry and analyses of optical spectra, may permit determinations of many constituents with little or no chemical preparation. Very little attention has yet been given to these methods.

4. The introduction of physical techniques and methods into the procedures for biochemical analyses may permit simplification of the procedures for some constituents.

5. Clinical technology today is generally not advancing as rapidly as the increase of medical knowledge and industrial technology might indicate. A primary reason for this lag has been insufficient research directed toward new applications of basic research and the development of new analytical techniques.

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ILLUSTRATIONS

TABLES

Section 1

INTRODUCTION

1.1 PROGRAM ADMINISTRATION

Hayes International Corporation, Birmingham, Alabama, under NASA Contract NASw-1560, has studied the feasibility of using physical methods to accomplish biochemical analyses under space flight conditions. The program was sponsored by the Space Medicine Directorate of the Office of Manned Space Flight, National Aeronautics and Space Administration. The technical representative for NASA headquarters was Dr. Sherman P. Vinograd, Director of Medical Science and Technology for the Space Medicine Directorate. The program monitor was Dr. Elliott Harris, Chief of Biochemical Research at the Manned Spacecraft Center, Houston, Texas.

The Hayes Program Manager was Dr. C. M. Askey, Chief of Research, and the Project Scientist was Ned D. Gilliam. The period of performance under the contract was 16 January - 16 September 1967. A group of eminent medical scientists from the University of Alabama Medical Center in Birmingham served as consultants and principal contributors to this study. These men, their fields of speciality, and their responsibility to this study were as follows:

Homer G. Biggs, Ph.D. Biochemistry	Amino nitrogen, BUN, Creatine, Creatinine, NPN, Total nitrogen, Bicarbonate, Chlorides, Phosphates, Pyrophosphates
G. William Cole, M.D. Hematology	Plasma thromboplastin component and other coagulation factors
Basil Doumas, Ph.D. Biochemistry	Fibrinogen, Hemoglobin, Methemoglobin, Mucoproteins, Proteins, Transferrins, Manganese, Sulfates, Zinc
Howard C. Elliott, Ph.D. Biochemistry	Adrenocorticotrophic hormone, Antidiuretic hormone, Catechol amines, Protein bound iodine, Serotonin, Thyroxine, Thyroxine binding prealbumin

Wells R. Moorehead, Ph.D. Biochemistry	Blood lactic acid, Sugar (glucose)
William Niedermeier, Ph.D. Biochemistry	Aldosterone, Alkaline phosphatase, Bilirubin, 17-Hydroxy corticosteroids, LDH isozymes, Calcium, Magnesium, Potassium, Sodium
Thomas A. Noto, M.D. Clinical Pathology	Immune bodies (complement and antibodies)
Kenneth M. Pruitt, Ph.D. Molecular Biology	General consultation
Jon V. Straumfjord, M.D., Ph.D. Clinical Pathology	Coordination and general consultation
Seymour S. West, Ph.D. Biomedical Engineering	Optical spectra
K. Lemone Yielding, M.D. Molecular Biology	Enzymatic reactions and general consultation
Edward L. Robinson, Ph.D. Nuclear Physics	Neutron activation

1.2 PURPOSE AND ORGANIZATION

The purpose of this study was to determine the feasibility of using physical methods for biochemical analyses under space flight conditions. The contract called for (1) a literature survey of industrial, Department of Defense, and academic sources with the submission of an annotated bibliography, and (2) a weighted evaluation of techniques and methods.

The literature survey, with which the program was initiated, included several automated searches by the National Library of Medicine, several by the Defense Documentation Center, inquiries to selected industrial sources, and a thorough search of Chemical Abstracts. The state of the art as revealed by the literature survey provided a meaningful context for evaluating new methods and variations or extensions of current methods for performing biochemical analyses. The limited duration of the contract and the time required to complete the literature survey, however, prevented as thorough an investigation of new methods as had been anticipated.

In the discussion of the various methods and techniques considered for the analysis of biochemical parameters, an attempt has been made to distinguish between currently proven and accepted methods, representing the present state of the art of clinical analysis, and extensions and variations of these methods which, along with new approaches to biochemical analysis, have not been fully explored and must be studied in more detail before designing flight instrumentation.

In studying the applicability of physical methods of analysis, one must realize that the distinction between physical and chemical methods is frequently a matter of degree. Most analytical methods require some chemical preparation (separation, reactions, etc.), and most methods employ physical measurement of the end point. The emphasis here has been on maximum reduction of wet chemistry.

Section 2

LITERATURE SEARCH

2.1 NATIONAL LIBRARY OF MEDICINE

The National Library of Medicine makes available to interested persons a literature search of journals abstracted in Index Medicus. This computerized search, known as Medical Literature, Analysis and Retrieval System (MEDLARS), is available only as far back as 1964, but it was felt that abstracts for these three years would provide a good introduction to recent work. Three search requests were submitted to the local MEDLARS office. The first request, submitted on 30 January 1967, asked for all references to analyses of body fluids for the biochemical constituents shown in Table 1. It also sought references to the use of physical methods for rendering biochemical analyses. A second request submitted at about the same time sought to determine what work had been done on the effects of space radiation (electromagnetic and particulate) on the parameters of Table 1. Because of a moratorium imposed on new MEDLARS searches by the National Library during December and January, these requests were not submitted by the local office until 24 February 1967. The returns from these requests were not received until 23 March 1967. Meanwhile, a third request was submitted relative to the analysis of all blood coagulation factors. The results of this third request were received about a month later.

At the time the MEDLARS searches were requested the format of the computer readout was not known, but a short abstract was believed to be included with each reference. When the returns were received, they contained only title, author, source, and key words. The absence of abstracts rendered the returns virtually useless, particularly in the remaining time. In the meantime, other literature searches had been initiated.

2.2 DEFENSE DOCUMENTATION CENTER (DDC)

At the same time the first MEDLARS searches were requested, similar requests were submitted to DDC. The first and second requests to DDC were

Table 1

CONSTITUENTS TO BE CONSIDERED FOR

PHYSICAL METHODS FOR BIOCHEMICAL ANALYSES

Creatine (S, U)	Pyrophosphates (U)
Creatinine (S, U)	NPN (S)
Proteins (S, U)	BUN (S)
Mucoproteins & related Biocolloids (S, U)	Sugar (WB)
Sodium (S, U, F, Sw)	Amino Nitrogen (S)
Potassium (S, U, F, Sw)	Total Nitrogen (U)
Chlorides (S, U, F, Sw)	Blood Lactic Acid (WB)
Phosphates (S)	Bilirubin (S)
Alkaline Phosphatase (S)	Protein Bound Iodine (PBI) (S)
Calcium (S, U, F, Sw)	17-Hydroxy Corticosteroids (U)
Magnesium (S, U)	Catecholamines (S)
Manganese (S, U)	Aldosterone (U)
Bicarbonate (S)	Antidiuretic Hormone (ADH) (S, U)
Zinc (S, U)	Serotonin (U)
Sulfates (S, U)	LDH Isozymes (S)
Thyroxine (S)	Transferrin (S)
Thyroxine Binding Prealbumin (TBPA) (S)	Hemoglobin (S, U)
Adrenocorticotrophic Hormone (ACTH) (S)	Methemoglobin (U)
Immune Bodies (Complement and Antibodies) (S)	Fibrinogen (U)
Plasma Thromboplastin Component (S)	

S = Serum or Plasma

U = Urine

WB = Whole Blood

F = Feces

Sw = Sweat

The same as those submitted to MEDLARS. The third request differed slightly. It requested data on flux densities and energy distributions of space radiation (again both electromagnetic and particulate), both inside and outside manned space capsules. Returns from the first two requests were received in about two weeks. As expected these requests produced very limited retrievals. The third request produced a bibliography of considerable length. The bibliographies, which included brief abstracts with each reference, were forwarded to the appropriate consultant for review and use if pertinent.

2.3 CHEMICAL ABSTRACTS

At the very beginning of the program the editorial office of Chemical Abstracts was contacted to determine whether or not computer searches of Chemical Abstracts were available. It was learned that computer tapes are currently being prepared, but automated searches are not expected to be available before 1969. At present the only means of reviewing Chemical Abstracts is manual. A very brief survey of subjects in Chemical Abstracts indicated that approximately fifty percent of the abstracts appeared in journals not abstracted by Index Medicus and hence would not be retrieved by MEDLARS. Furthermore, since MEDLARS covers only three years, some fifty-eight years of the literature abstracted by Chemical Abstracts would remain unreviewed without the manual search. At the first meeting with the consultants the matter of conducting a manual search of Chemical Abstracts was discussed at length. The consensus was that a complete review of Chemical Abstracts was requisite to adequately treat the problem. A system was set up to accomplish the formidable task of ferreting out every reference to the thirty-nine constituents in Table 1 plus related terms and reviewing every abstract relevant to the problem of applying physical methods to biochemical analyses.

Those pages of the cumulative indices which contained references to the thirty-nine biochemical constituents or related terms were xerographically copied and sent to the appropriate consultant. The consultant reviewed the indices, marking all abstracts he thought might be of interest. A graduate student or medical technologist assigned to assist the consultant retrieved the abstracts and read them. If he found that an abstract contained pertinent information on analytical techniques or instrumentation, he had it xerographically copied and submitted it to the consultant for further review. The index copying began with the volumes for 1906 and was carried through 1966. If an assisting searcher found an additional term which he considered important, the indices were checked and copied beginning with 1966 and proceeding backward to 1906 or until the term disappeared. During the course of the program specific papers were requested and copied. The total number of pages copied exceeded 9600.

2.4 INDUSTRY

From time to time during the program specific requests for literature references or reports were sent to selected individuals in the biomedical industry. In particular Dr. Robert Phillips, Vice President of G. K. Turner Associates, Palo Alto, California, manufacturers of fluorometric instruments, was contacted, apprised of the problem, and asked to send any pertinent reports or procedures that were available. He responded promptly and generously.

A similar request was sent to Dr. Gerald Soffen of the Jet Propulsion Laboratory at Pasadena, California. A copy of the Pace/Rho experiment[*] for analyzing animal urine in orbit was obtained.

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[*] See, for example, Biosatellite Project Document 883-12-06 (19 December 1966) and Biosatellite Fluorometry Experiment, JPL Technical Report (6 February 1967.)

Section 3

PHYSICAL METHODS OF ANALYSIS

3.1   INTRODUCTION

At the beginning of the program the biochemical constituents of Table 1 were divided into six somewhat arbitrary groups: proteins, protein metabolites, carbohydrates, steroids and enzymes, hormones, and inorganics. Each group was assigned to a consultant who became responsible for reviewing and evaluating the methods for each constituent in the group. Some subdivision of the groups was introduced during the early part of the program, and additional consultants were brought into the program. To provide a uniform format for the evaluation of analytical methods, the following outline was developed:

A.   A brief statement of the principles of each significant method of analysis.

B.   An evaluation of the suitability of analytical methods for use under space flight conditions.

   1.   An evaluation of some fourteen merit parameters by assigning to each a rating between zero and ten, with zero representing the least desirable rating value and ten, the most desirable.

   2.   A discussion of the merit table and the recommended techniques.

C.   A discussion of areas deserving further research and development.

D.   An annotated bibliography.

The merit table, as indicated above, presents some fourteen parameters, three of which are given actual values, while the remaining eleven are rated from zero to ten. The last item in the merit table is a Figure of Merit, which is calculated from the ratings assigned to the preceding eleven parameters and is intended to indicate the relative suitability of the method for use under space flight conditions. The Figure of Merit is calculated according to the formula

$$M = \frac{R}{4} \, (1 + 0.1\, G) \, (1 + 0.1\, S) \, \frac{\Sigma \, w_i \, P_i}{\Sigma \, w_i},$$

where R is the reproducibility, G is the suitability to null gravity, S is the

overall safety, and w_i is a weight attached to the merit parameter P_i. The values of the weights are listed in Table 2. The multiplicative factor 1/4 is a normalizing constant to provide a maximum value of 100 for the Figure of Merit. During the course of evaluating the various methods it was found that some merit parameters could not be quantitated for lack of information. In such cases a merit range is shown which corresponds to the range of the Figure of Merit with the indeterminate parameters set first to zero and then to ten. The mean Figure of Merit assumes a value of 0.5 for all indeterminate parameters.

Table 2

WEIGHTS ATTACHED TO MERIT PARAMETERS

Nontoxic reagents	1.0
Noncaustic reagents	1.0
Applicability*	0.8
Specificity	0.8
Insensitivity to environmental change	0.6
Reagent volatility*	0.6
Ease in training personnel	0.4
Degree of separation required (10=none)	0.4
Minimal handling by analyst	0.4
Common use of analytic equipment	0.2
Nondestructive of sample	0.2

* Not included in all merit tables

3.2 SUMMARY

In order to permit comparison of the best current methods of analysis, a summary is provided in Table 3. The order of the constituents has been

Table 3

BEST CURRENT METHOD FOR EACH DETERMINATION

Class.		Constituent	Method	Figure of Merit	Page
PROTEINS	Enzymes	Alkaline phosphatase	Phenolphthalein phosphate (Spectrophotometric)	59	11
		LDH isozymes	Electrophoretic separation	34	15
		Coagulation factors	Photometer Turbidimetric	39*	32
		Fibrinogen	Martinek	70	39
		Blood hemoglobin	Oxyhemoglobin	74	43
		Plasma hemoglobin	Martinek	42*	48
		Urine hemoglobin	Dip stick	87	52
		Immune bodies	Electrophoresis	29	56
		Methemoglobin	Martinek	59	71
		Mucoproteins	Protein-bound hexose	9*	75
		Serum albumin	Bromcresol green	75	78
		Total serum proteins	Refractometry	82	81
		Transferrin	Williams and Conrad	55	86
		Urinary proteins	Kutter (dip stick)	48*	91
STER.	Hormones	Adrenocorticotrophic hormone	None		94
		Antidiuretic hormone	None		96
		Aldosterone	Gas chromatography	3	98
		17-Hydroxycortico-steroid	Isotope dilution	57	100
NITROGENOUS COMPOUNDS		Catecholamines	"VMA" colorimetric	34*	107
		Serotonin	5-HIAA	4	110
		Thyroid function	Total thyroxine	22*	113
	Protein metab.	Amino nitrogen	β-Napthoquinonesulfonate	20*	119
		Blood urea nitrogen	Urease	48*	122
		Nonprotein nitrogen	Modified Kjeldahl	11*	130
		Total nitrogen	Modified Kjeldahl	11*	130
		Bilirubin	Iceterus index	34*	133
		Creatine	Modified Jaffe	14*	138
		Creatinine	Jaffe	29*	138
CARBO		Lactic acid	Modified Loomis-Noll	79	144
		Glucose	Glucose oxidase	73	148
INORGANICS		Bicarbonate	Electrometric assay	65*	153
		Chlorides	Electrometric assay	68*	156
		Inorganic phosphate	Colorimetric	14*	158
		Inorganic pyrophosphate	Colorimetric	8*	158
		Manganese	Fernandez	7*	161
		Sodium, potassium, magnesium, calcium	Neutron activation	64	165
		Sulfates	Miller	36	168
		Zinc	Johnson	14*	172

*This figure represents a mean value of the Figure of Merit. Refer to page indicated. N.B. Care should be exercised in comparing

altered somewhat since the original grouping at the beginning of the program. The constituents have been divided into five major categories according to structure -- proteins, steroids, nitrogenous compounds, carbohydrates, and inorganics. Three minor categories group constituents with functional similarities -- enzymes, hormones, and protein metabolites. For methods having a merit range the mean figure of merit has been listed.

3.3   CURRENT ANALYTICAL METHODS

3.3.01 Alkaline phosphatase in blood serum

A.   Principles of present methodology

1. Phenolphthalein phosphate method.[4, 5] (Spectrophotometric)

To a solution of phenolphthalein phosphate at pH 9.0 is added 0.2 ml. of blood serum. The solution is incubated exactly 30 minutes at 37° C during which time the enzyme present in the blood serum hydrolyzes the substrate, releasing free phenolphthalein which has a strong absorbance at 550 mμ. The reaction is first order and the absorbance at 550 mμ is proportional to enzyme concentration. The reagents have been prepared in the form of a stable compressed tablet to which only buffer solution and blood serum need be added to perform the test.[8]

2. Phenolphthalein phosphate method (Visual)

The test is performed as above, except that the color produced is compared with a color chart supplied by Warner-Chilcott, Moris Plains, N.J.

3. Test paper method

When mono alpha naphthylphosphoric acid (I) is treated with alkaline

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the figures of merit for various determinations. Because of the subjective aspect of evaluating analytical methods, the resultant figures of merit may not be absolutely consistent throughout the list; however, within a given category of constituents the figures of merit should give a reliable indication of the relative overall suitability of the methods for use in space.

phosphatase, alpha naphthol is liberated which will react with diazo-o-anisidine (II) to produce a compound with a blue violet color. The preparation of a test paper which consists of filter paper impregnated with I and II has been described.[7] The intensity of color produced when a drop of blood serum is placed on the test paper and incubated at room temperature in an atmosphere saturated with water vapor is compared with a standard color chart.

B. Applicability of present methodology to space flight conditions

1. Merit table

Alkaline Phosphatase in Serum

Merit Parameters	Phenolphthlein phosphate		
	Spectrophotometric	Visual	Test Paper
1. Sensitivity	7. 5 Bodansky u/ml	7. 5 Bodansky u/ml	unknown
2. Sample size	0. 2 ml.	0. 2 ml.	1 drop
3. Time required	15 min.	12 min.	5 min.
4. Reproducibility	10	5	5
5. Suitability for null gravity use	4	6	10
6. Overall safety	10	10	10
7. Nontoxic reagents	10	10	10
8. Specificity	10	10	10
9. Insensitive to environmental changes	10	10	10
10. Ease in training personnel	5	9	10
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	4	8	10
13. Common use of analytic equipment	10	8	8
14. Nondestructive of sample	0	0	0
15. Figure of merit	59	36	47

2. Discussion

Several reports attest to the simplicity and reliability of both the spectrophotometric and visual methods using phenolphthalein phosphate substrate.[1,3,6] Although the visual comparison gives only semiquantitative results, it appears to be adequate for diagnostic purposes. The procedures are generally accepted and diagnostic values are well established by these methods. Results obtained by the test paper method are less well documented. In view of its simplicity, further investigation of this method appears to be indicated. The application of test papers and compressed tablets to the problem of laboratory diagnosis has been discussed.[2]

C. Promising areas for research and development

The methods described appear adequate for clinical purposes and are sufficiently simple that adaptation to the space environment should pose no formidable problems. One promising area for future development would be thermal analysis based on the heat of reaction involved in hydrolysis of the substrate by alkaline phosphatase.

D. References

1. Babson, A.L.; Read, R.A.; Phillips, G.E.; Luddecke, H.F.; Use of a New Assay in Study of Serum Alkaline Phosphatase Levels in 2,000 Hospital Patients. Clin. Chem., Vol. 6, 1960, pp. 495 - 500.

   Describes application of commercially available tablet (Phosphatabs)* for routine use in clinical laboratory. Results were found to be reliable and in agreement with those obtained by another established procedure.

2. Drevon, B.; Accelerated Biological Analysis; Use of Tablets and Reactive Papers; General Considerations. Ann. Biol. Clin., Vol. 21, 1963, pp. 453 - 456.

   Discusses problems of application of tablets and reactive papers to the search for abnormal substances in urine.

3. Feldman, P.E.; A New Method for Alkaline Phosphatase Level Determinations. Am. J. Med. Tech., Vol. 25, 1959, pp. 143 - 144.

Results using "Phosphatabs"* were found to be reliable in both jaundiced and nonjaundiced patients. Test required 12 minutes to complete.

4. Hovels, O.; Laun, M.; On Determination of Alkaline Phosphatase Activity in Small Amounts. Z. Kinderheildt, Vol. 71, 1951, pp. 357 - 368.

   Describes a micromethod based on the method of Huggins and Talalay using phenolphthalein phosphate as substrate. Method requires 0.05 ml of blood serum.

5. Huggins, C.; Talalay, P.; Sodium Phenolphthalein Phosphate as a Substrate for Phosphatase Tests. J. Biol. Chem., Vol. 159, 1945, pp. 399 - 410.

   Describes a synthesis of the substrate and its application to determination of alkaline phosphatase. Simplicity and accuracy are discussed.

6. Klein, B.; Read, P.A.; Babson, A.L.; Rapid Method for Quantitative Determination of Serum Alkaline Phosphatase. Clin. Chem., Vol. 6, 1960, pp. 269 - 275.

   Describes use of a compressed tablet and direct photometric measurement of phenolphthalein released from phenolphthalein phosphate. As many as 30 determinations were completed per hour.

7. Menske, R.; Method for the Rapid Detection of Alkaline Phosphatase. Naturwissenschaften, Vol. 46, 1959, pp. 668 - 669.

   Describes the preparation and use of a test paper for semiquantitative determination of alkaline phosphatase in blood serum which gives clinically useful results.

8. Warner-Lambert Pharmaceutical Co. Serum Alkaline Phosphatase Diagnostic Preparation. Brit. Patent 863739, March 22, 1961.

   Describes the preparation of a compressed tablet which contains phenolphthalein phosphate, magnesium sulfate and tris buffer which provides a simple method for determining alkaline phosphatase in blood serum.

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* Trademark of Warner-Chilcott, Morris Plains, N.J.

3. 3. 02 <u>Lactate Dehydrogenase (LDH) Isozymes</u>

   A. Principles of present methods

      1. Electrophoretic separation of isozymes.

      2. React separated isozymes with a tetrazolium salt in presence of sodium lactate, phenazine methosulfate and niacin adenine dinucleotide (NAD).

      3. Fix strip in an aqueous solution of 50% methanol and 10% acetic acid.

      4. Determine intensity of reduced tetrazolium with a scanning micro-densitometer similar to the Analytrol.*

   B. Applicability of present methods to space flight conditions

      1. Merit table

<div align="center">LDH Isozymes</div>

Merit Parameters	
1. Sensitivity	-
2. Sample size	5 μ1
3. Time required	2 hrs.
4. Reproducibility	8
5. Suitability for null gravity use	8
6. Overall safety	8
7. Nontoxic reagents	5
8. Specificity	10
9. Insensitive to environmental changes	5
10. Ease in training personnel	5
11. Degree of separation required (10=none)	0
12. Minimal handling by analyst	5
13. Common use of analytic equipment	5
14. Nondestructive of sample	0
15. Figure of merit	34

      2. Discussion

          The present state of the art requires separation of isozymes by

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* Spinco Division, Beckman Instruments, Palo Alto, California

some physicochemical method prior to their quantitation. Electrophoresis on cellulose acetate appears to be the most satisfactory method for accomplishing this.[7,12] An electrophoretic cell similar to that manufactured by the Millipore Corporation[*] appears adaptable to the space environment. In this apparatus buffer soaked sponges serve as electrode vessels. Other electrophoresis media have been used for separation of LDH isozymes. These include Sepraphore III,[10] agar gel,[8,15] and starch gel.[1] Of these, Sepraphore III[**] which is a modified cellulose acetate might afford separation of the isozymes in less time than is required on cellulose acetate. The use of higher potentials might achieve the same end. Thin layer chromatography on DEAE Sephadex[***] has also been used for separating LDH isozymes.[4]

The color reagent is added to the electrophoretic strip from a second cellulose acetate strip which has previously been impregnated with the reagent.

The choice of tetrazolium salts for the color reagent and the choice of operating conditions to obtain optimal results have been discussed.[13] Experiences in the clinical laboratory have been described.[9] Spectrofluorometric[8] and spectrophotometric[15] methods of detection have also been described.

C. Promising areas for research and development

Several reports indicate that the LDH isozymes have different heats of inactivation.[5,11,16] Development of a method of analysis based on these properties and on sensitivity to inhibitors[6,14] would obviate physicochemical separation of the isozymes. Recent advances indicate, for example, that a method based on heat sensitivity may soon be available for routine laboratory use.

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[*] Millipore Filter Corp., Bedford, Mass.
[**] Registered trademark of Gelman Instrument Co., Ann Arbor, Mich.
[***] Registered trademark of Pharmacia, Inc., Stockholm, Sweden

D. References

1. Allison, M. J.; Gerszten, E., Sanchez, B.; A Method for Quantitation of Lactic Dehydrogenase in Vertical Starch Gels. Tech. Bull. Registry Med. Tech., Vol. 33, 1963, pp. 165 - 167.

    Separated isozymes on starch gel and determined activity with phenazine methosulfate. Quantitation was achieved with a microscope photometer.

2. Barnett, H.; Electrophoretic Separation of Lactate Dehydrogenase Isozymes on Cellulose Acetate. Biochem. J., Vol. 84, 1962, pp. 83 - 84.

    Identified five well defined bands on electrophoretic patterns with LDH activity.

3. Clarke, J. T.; Simplified Disk (polyacrylamide gel) Electrophoresis. Ann. N. Y. Acad. Sci., Vol. 121, 1964, pp. 428 - 436.

    Separated isozymes on polyacrylamide gel and determined enzyme activity with Nitro Blue Tetrazolium.

4. Determan, H.; New Applications of Thin Layer Chromatography. I Chromatography on Dextran Gel. Experientia, Vol. 18, 1962, pp. 431 - 432.

    Effectively separated isozymes on DEAE Sephadex in a simple gradient chamber.

5. Dubach, U. D.; Vareokojic; The Diagnostic Significance of the Lactic Dehydrogenase Isozymes (Heat Inactivation Method). Schweiz. Med. Wochschr., Vol. 93, 1963, pp. 1196 - 1200.

    Studied heat inactivation of LDH isozymes in 300 patients and correlated results with clinical diagnosis.

6. Fritz, P.; Rabbit Lactate Dehydrogenase 5: A Regulatory Enzyme. Science, Vol. 150, 1965, p. 364.

    Oxalacetate activated LDH 5 at low concentrations and inhibited at high concentrations, whereas LDH 1 was only inhibited.

7. La Mancusa, S. J.; Romel, W. C.; Electrophoresis of Lactic Dehydrogenase Isozymes in Serum on Cellulose Polyacetate. Lancet, Vol. 1, 1964, p. 668.

A simplified method is described.

8. Laursen, T.; Electrophoresis of Serum Lactic Dehydrogenase on Agar Gel with Fluorimetric Determination of the Enzyme Activity. Scand. J. Clin. Lab. Invest., Vol. 14, 1962, pp. 152 - 156.

Describes electrophoretic separation of isozymes on agar gel followed by fluorimetric determination of activity with the diphosphopyridine nucleotide-pyruvate reaction.

9. Mager, M.; Blatt, W.F.; Abelmann, W.H.; The Use of Cellulose Acetate for Electrophoretic Separation and Quantitation of Serum Lactic Dehydrogenase Isozymes in Normal and Pathological States. Clin. Chim. Acta. Vol. 14, 1966, pp. 689 - 697.

Describes experiences with the method in a clinical laboratory. A discussion of the diagnostic significance of the results is given.

10. Opher, A.W.; Collier, C.S.; Miller, J.M.; Rapid Electrophoretic Method for the Determination of the Isozymes of Serum Lactate Dehydrogenase. Clin. Chem., Vol. 12, 1966, pp. 308 - 313.

A convenient procedure for the separation and quantitation of LDH isozymes is described. It used polyacetate Sepraphore III for separation and tetranitro blue tetrazolium followed by densitometry for quantitation.

11. Paunier, L.; Rotthauwe; Determination of Serum Lactic Dehydrogenase Isozymes by Relative Heat Stability Test. Enzymol. Biol. Clin., Vol. 3, 1963, pp. 87 - 96.

Found that 20% of LDH activity was lost following incubation of serum at 57° C for 30 minutes. Noted differences in serum from diseased subjects.

12. Preston, J.A.; Briere, R.O.; Batasakis, J.G.; Rapid Electrophoretic Separation of Lactate Dehydrogenase Isozymes on Cellulose Acetate. Am. J. Clin. Pathol., Vol. 43, 1965, p. 256.

Gives a complete description of the method recommended in the present report.

13. Starkweather, H.H.; Spencer, H.H.; Schwartz, E.L.; Schach, H.K.; Electrophoretic Separation of Lactate Dehydrogenase Isozymes and Their Evaluation in Clinical Medicine. J. Lab. Clin. Med., Vol. 67, 1966, pp. 329 - 343.

Describes a procedure for controlled separation of the isozymes on agar gel and discusses conditions for accurate quantitation. A discussion of the diagnostic significance of the results is given.

14. Vesell, E.S.; Yielding, K.L.; Effects of pH, Ionic Strength, and Metabolic Intermediates on the Rates of Heat Inactivation of Lactate Dehydrogenase Isozymes. Proc. Nat. Acad. Sci., Vol. 56, 1966, p. 1317.

LDH 1 and 5 showed differential heat lability and were protected in a differential manner by metabolic intermediates.

15. Wieme, R.J.; van Sande, M.; Karcher, D.; Lowenthall, A.; van der Helm, J.; A Modified Technic for Direct Staining with Nitro-Blue Tetrazolium of LDH Isozymes in Agar Gel Electrophoresis. Clin. Chim. Acta., Vol. 7, 1962, pp. 750 - 754.

Separated isozymes on agar gel and stained with tetrazolium blue. Obtained a linear response over the range of 2 - 40 Buchler units/ml.

16. Wuest, H.; Isozymes of Lactic Dehydrogenase in Clinical Diagnosis; Methods of Thermal Inactivation. Med. Welt, 1965, pp. 1798 - 1800.

Found that LDH 1 was stable at 65° for 30 minutes, but LDH 5 was labile under these conditions. Correlated results with clinical observations.

3.3.03 Coagulation factors

A. Introduction

The only coagulation factors listed in Table 1 were plasma thromboplastin component and fibrinogen; however, conversations with NASA indicated that the real interest was in coagulation in general. Consequently, a more comprehensive study of coagulation factors and their analysis in vitro was undertaken.

Coagulation studies are readily justified in space principally because of the radiation hazard. Radiation in experimental animals as well as humans has been shown to alter a variety of coagulation factors.

A list of coagulation factors is given in Table 4. These factors circulate in an inactive state, and are for the most part either proteins or lipoproteins. Most are enzymes which can be accurately measured only by means of their activity in coagulation. Reliable assay is limited to their ability to accelerate clot formation. Fibrinogen is the sole exception.

Figure 1 demonstrates how these factors interact to form a fibrin clot. Coagulation is divided into four phases:

1. Thromboplastin generation
2. Prothrombin conversion to thrombin
3. Fibrinogen conversion to fibrin clot
4. Fibrinolysis or clot lysis

Phase I or thromboplastin generation is concerned with the formation of a substance (thromboplastin) which will accelerate the conversion of prothrombin to thrombin. It is an enzyme or aggregation of enzymes which, in the presence of calcium, is required for optimal prothrombin conversion. Thromboplastin formation is initiated when blood comes in contact with a foreign surface (i.e. glass). Glass will activate Factors XII and XI. These in turn activate the enzymes Factors V, VII, VIII, IX and X. Maximal thromboplastin generation is accomplished only when
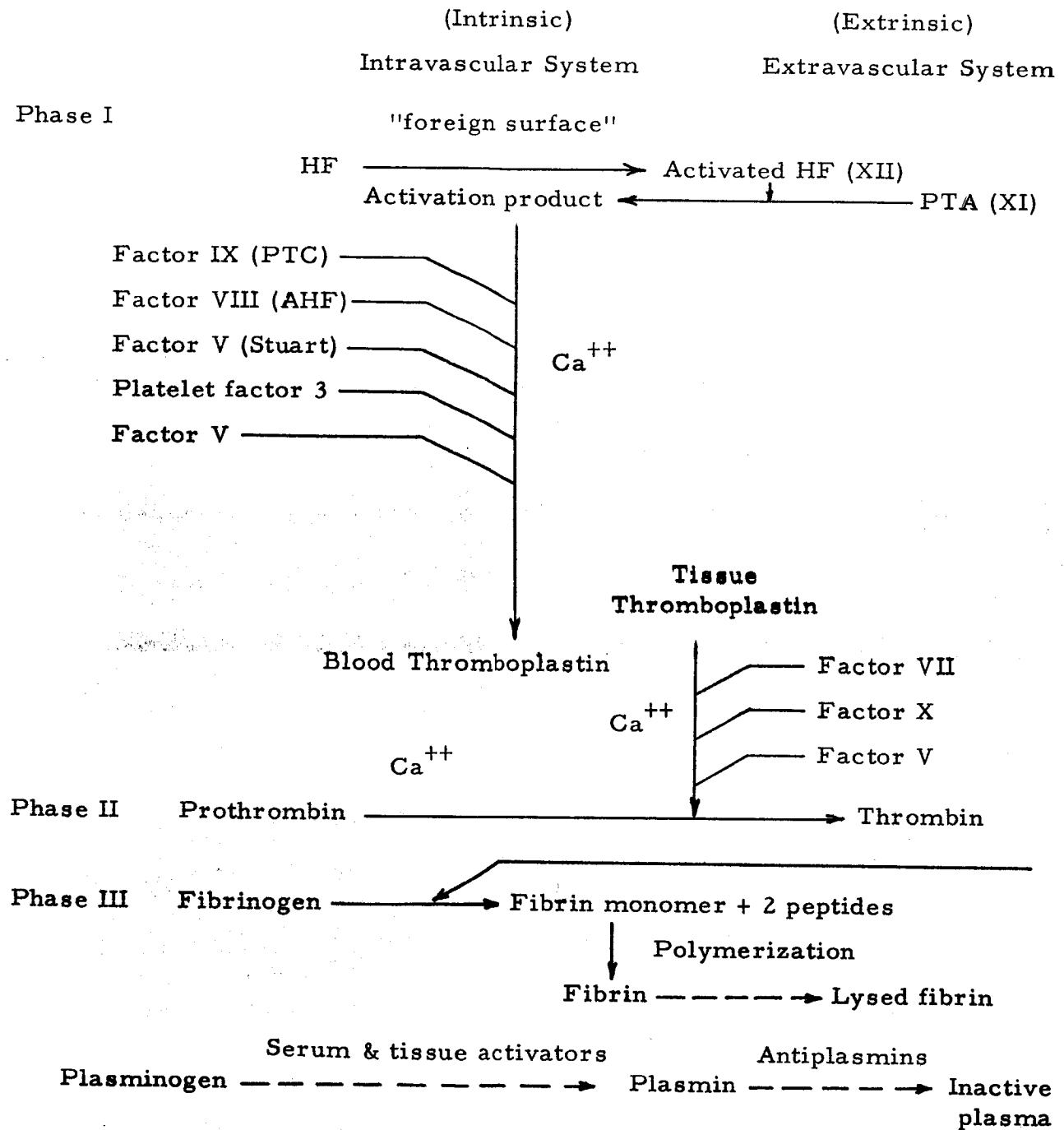
Figure 1. Interaction of Factors in Formation of Fibrin Clot.[2]

there are optimal amounts of these enzymes present.

Phase II or prothrombin conversion to thrombin will occur max-imally if there are sufficient quantities of prothrombin and thrombo-plastin present. Calcium is an additional requirement.

Table 4

COAGULATION FACTORS

Factor	I =	Fibrinogen
Factor	II =	Prothrombin
Factor	III =	Thromboplastin
Factor	IV =	Calcium
Factor	V =	Labile Factor, Proaccelerin, Plasma Ac-globulin
Factor	VI =	Active Factor V, (Accelerin)
Factor	VII =	Stable Factor, Autoprothrombin I, SPCA
Factor	VIII =	Anti-Hemophiliac globulin (AHG) or Factor (AHF), Hemophilia A, Platelet Co-factor I
Factor	IX =	Plasma Thromboplastin Component (PTC), Auto-prothrombin II, Christmas Factor, Hemophilia B
Factor	X =	Stuart-Power Factor
Factor	XI =	Plasma Thromboplastin Antecedent (PTA), Contact Factor
Factor	XII =	Hageman Factor, Contact Factor

Platelet Factor or Factors

Tissue Thromboplastin = Incomplete Thromboplastin

Blood Thromboplastin = Complete Thromboplastin

Phase III or fibrin formation is dependent upon optimal quantities of thrombin and fibrinogen present. Thrombin is the active enzyme which splits the fibrinogen molecule in such a fashion that fibrin monomers are formed. These fibrin monomers align in an end to end and side to side manner so that a fibrin gel or clot is formed. Phase IV or fibrinolysin activity occurs under certain circumstances. Fibrinolysin or plasmin has a great affinity for fibrin. The fibrinolysin which is bound to the fibrin clot

during the process of coagulation exists usually in an inactive state (plasminogen). Certain factors will enhance the conversion of plasminogen to plasmin (fibrinolysin). The clot will undergo lysis when this occurs.

The in vitro study of coagulation is composed of tests which measure part of a single phase, a single phase, or a group of phases. There is no single test which will measure all phases.

The methods chosen, with their modifications are potentially suitable for the study of blood coagulation in space for the following reasons:

    a. Minimal hazard to the physiological well being of the astronauts.

    b. Limitation in laboratory space and equipment.

    c. Minimal degree of technical skill and proficiency required.

    d. Reliability and reproducibility of the methods are reasonably good.

    e. Minimal reagents required.

    f. No toxic chemicals are used.

    g. Minimal influence on other tests to be performed.

    h. Adaptability to null gravity environment.

In studying blood coagulation in vitro it is requisite that certain precautions be taken if reproducible results are to be obtained. Among them are the following:

1. Blood collection

    A uniform and rapid method of collection is one of the most important aspects of coagulation study. The problem is mainly that of "contact activation." This problem can be overcome in several ways.

    a. Collect blood in non-wettable vehicles such as siliconized glassware or smooth plastic containers.

    b. Anticoagulation

    c. Performance of tests immediately upon collection.

2. Anticoagulants

Sodium citrate or sodium oxalate are the only completely satis-factory anticoagulants. These chemicals bind calcium in such a fashion that coagulation is prevented and thromboplastin generation is inhibited. EDTA and heparin are not suitable or readily adaptable.

3. Temperature

Maximal acceleration of coagulation occurs at 37° C. However, coagulation reactions may be performed in a temperature range of 20 - 40° C. Tests may not be as reproducible at other temperatures but are workable. The temperature of the reaction must be the same each time the test is performed.

4. pH

Coagulation reactions are maximally reactant at a pH of 7.2 to 7.4. Very little latitude is possible. Blood must remain in a sealed container until ready for testing. This prevents the loss of CO_2 and tends to limit pH changes. The tests should be performed within two hours after collection if blood is kept at room temperature or sodium oxalate is used as an anticoagulant. Refrigeration at 4° C or sodium citrate as an anticoagulant permits a longer interval between collection and testing.

5. Fibrin endpoint

Several methods of coagulation study have been devised in recent years which measure several of the coagulation components by means other than a fibrin clot as an endpoint of the reaction. These methods are unproven and not desirable. For the purposes of this program the physiology of clotting is of greater interest than the biochemistry Biochemical determinations that do not use fibrin end-point formation are difficult to correlate with in vivo coagulation. Therefore, the proposed methods are dependent upon a fibrin endpoint.

6. Duplicate testing and controls

There are no tests in coagulation which are so reproducible that

reliance can be placed wholly upon one determination. Duplicate and sometimes triplicate determinations are required for meaningful interpretation. Normal control plasma is generally employed as the method of assuring the realibility of a coagulation test. There are few suitable other standards.

7. Mixing of reagents

Interpretation and reliability of the methods are dependent to a great extent on uniform and rapid mixing of the reagents with patient plasma or blood.

Coagulation cannot be studied unless these requirements in whole or at least the greater part can be controlled.

In principle it is possible to study coagulation using either whole blood or plasma; however, plasma is preferable for two reasons. A fibrin endpoint is easier to detect, and inadvertent hemolysis of erythrocytes can be seen. Hemolyzed red cells alter the results of coagulation studies and yield uninterpretable results. The use of plasma permits more reproducible results. The four phases of coagulation and methods for studying them follow:

B. Principles of present methodology[1, 2, 3, 4, 5, 6]

1. The study of Phase I of coagulation (thromboplastin generation) in order of preference (only one required).

a. Activated partial thromboplastin time

This test measures moderate to severe changes in those factors (V through XII) concerned with thromboplastin generation. Severe depletions of prothrombin and fibrinogen will also prolong the clotting time. Minor changes in these factors will not be measured in this test. It is unlikely that clinical bleeding will occur, secondarily to a depletion of these factors, if this test is normal. It will not detect a deficiency of Factor VII or platelets.

Method: 0.1 ml plasma required

Cephalin (phospholipid platelet substitute), $CaCl_2$, kaolin or celite particles, and plasma are mixed together. The

time is recorded for clot formation. Normal time is under 45 seconds.

Reagents for this test are preserved in a dry or liquid form. The dried powder may be reconstituted in distilled water. Cephalin activity may be affected by temperature change. It is best preserved at 4° C. Lyophyllized control plasma would be desirable as a check on technique and reagent stability. If this test should become abnormally prolonged during space flight, the following procedures will further delineate the problem.

Three additional reagents are required.

(1) Lyophillized aged normal serum contains Factor IX and X activity. It does not have Factor V and VIII activity. Correction of a prolonged partial thromboplastin time by the addition of this serum to a patient plasma would indicate a deficiency of Factors IX or X. The Quick one stage prothrombin time (See Method for Phase II) will be prolonged by a Factor X deficiency but not by a Factor IX deficiency.

(2) Lyophillized $BaSO_4$ adsorbed plasma contains Factor V and VIII activity. It does not contain Factor IX or X activity. Correction of a prolonged partial thromboplastin time by adsorbed plasma would indicate a deficiency of Factor V or VIII. The Quick one stage prothrombin time is prolonged by Factor V deficiency but not prolonged by a Factor VIII deficiency.

(3) If both normal serum and adsorbed plasma correct the patient's plasma defect then depression of either Factor XI or XII would be expected. Both serum and adsorbed plasma contain these factors.

Fowl plasma has Factor XI activity but not Factor XII activity. Correction of the partial thromboplastin time with fowl plasma

would indicate a deficiency of Factor XII.

b. Alternative methods for Phase I

(1) Activated whole blood recalcification time

This method measures the same factors of coagulation as does the activated partial thromboplastin time. Two-tenths milliliter of anticoagulated blood is mixed with $CaCl_2$ and kaolin or celite particles. The time is recorded for clot formation. Normal: 90 - 150 seconds. Similar reagent substitutions might be used if desired in order to correct a prolonged clotting time. This would require considerable investigation in order to document the reliability and reproducibility.

(2) Capillary clotting time for Phase I

This test measures the same factors in coagulation as the activated partial thromboplastin time and the whole blood recalcification time. It is less reproducible for several reasons. Contact activation is not uniform and blood obtained by finger stick is contaminated by tissue juices. The latter may factitiously accelerate coagulation.

Blood is aspirated or allowed to flow into a 10 cm capillary tube. Every 30 seconds a segment of the tube is broken and the ends examined for fibrin thread. Normal time is 2 - 4 minutes.

2. The study of Phase II (prothrombin conversion to thrombin) by means of the Quick one stage prothrombin time method [7, 8, 15]

a. In plasma

This test is a measure of the prothrombin content of the blood. It is also a sensitive measure of Factors V, VIII and X activity. Severe depressions of substrate fibrinogen in patient plasma will also cause a prolonged prothrombin time. This test does not measure Factor VIII, IX, XI, XII, and platelet activity. Deficiences of these latter factors will go undetected if this method of measurement is used alone. It is preferable to the activated partial throm-

boplastin time when either prothrombin, Factor V or X deficiency are suspected, and is the only coagulation test that measures Factor VII activity.

Method: 0.1 ml plasma required

A small amount of $CaCl_2$ - thromboplastin mixture is added to the patient plasma, and the time is recorded for clot formation. Normal = 12 seconds. Tissue thromboplastin (an extract of animal brain or lung) contains Factor VIII, IX, XI, XII and platelet activity. Therefore, part of the first phase of coagulation is bypassed. **Tissue thromboplastin combines with plasma Factors V, VII and X to form blood thromboplastin (complete thromboplastin). Prothrombin is then converted to thrombin which in turn converts plasma fibrinogen into a fibrin clot.**

The reagents needed are an anticoagulant, $CaCl_2$ and **tissue thromboplastin.** Tissue thromboplastin is a thermolabile substance and requires refrigeration at 4° C for optimal activity (even in a lyophyllized state). A lyophyllized plasma control should be available (also thermolabile) that will serve as a check on reagents and technique.

b. **Whole blood**

These methods could be adapted to whole blood. Reliability and reproducibility would have to be further investigated.

3. **Phase III** [*] (Fibrinogen conversion to fibrin clot) [9]

a. Plasma - thrombin addition test

Plasma fibrinogen will convert maximally to fibrin in the presence of an optimal amount of thrombin. The rapidity of clot formation and the stability of the formed clot will roughly correlate with the fibrinogen concentration. A solid clot will form in a few sec-

_____
[*] For chemical analysis of fibrinogen, see section 3.3.04.

onds following the addition of thrombin. Poor clot formation and
stability occurs when the fibrinogen concentration falls below
100 mg%. Fibrinogen deficiency of lesser grades of severity will
not result in clinical bleeding. Two-tenths millileter of plasma
is required. This test does not measure other coagulation factors.
A control fibrinogen (100 mg%) or a series of fibrinogen concentra-
tions will assist the quantitation of the results.

b. Whole blood - thrombin addition test

The plasma method is probably easily adaptable to whole blood
without much impairment of accuracy or reproducibility.

4. Phase IV - Fibrinolysis [10,11]

Clot lysis may be measured by a very simple method. A tube of
blood is allowed to clot, and the clot is observed for clot lysis. How-
ever, differentiation between hypofibrinogenemia and elevated fibrino-
lysin activity is difficult. The following method is suggested.

a. Plasma - A heat-denatured fibrin plate or tube is required. A drop
of patient plasma is added to this media. The fibrin plate is ob-
served for lysis. The degree of lysis is roughly quantitative to the
the amount of fibrinolysin present. Normally minimal lysis occurs
over a period of several hours.

b. Whole blood - It is quite probable that the above method could be
adapted for whole blood, but again will require special investigation.

5. Platelets

Platelets, although important in hemostasis, are generally not pre-
dictably important in blood coagulation. The ability of a clot to retract
in a test tube is often proportional to the number and function of plate-
lets present. This is not a good method to quantitate minor to moderate
depression in platelet numbers. Therefore the following methods are
suggested.

a. Indirect platelet count

An estimate of platelet numbers may be made by microscopic

examination of a blood smear. Reliability would be $\pm$ 20% of actual platelet numbers. The obvious disadvantage is that a microscope is required.

b. Adenosine diphosphate (ADP)[17, 18, 19] aggregation test

A few micrograms of ADP are added to a small volume of plasma. A 10X ocular is used to examine the platelet aggregation in the plasma. Deranged platelet function will be indicated by a failure of visible platelet aggregation.

It may be possible to correlate the degree of aggregation with platelet numbers. This test then would obviate the need for a microscope.

c. The use of plasma or whole blood would be determined by choice of the above methods. It may be possible to adapt the platelet aggregation test to whole blood.

6. Reagents and equipment

The following reagents and equipment would be required to perform the foregoing experiments.

a. Reagents

1. Sodium citrate of sodium oxalate
2. $CaCl_2$
3. Distilled H_2O
4. Control plasma
5. Fibrinogen
6. Cephalin
7. Tissue thromboplastin
8. Thrombin
9. Adenosine diphosphate (ADP)
10. Kaolin or celite

(Items 4 - 10 may be lyophyllized).

C. Applicability of present methodology to space flight conditions

1. Merit table

This table is applicable for all Coagulation Fractions except platelets. (This table appears on the following page.)

2. Discussion

A fibrin endpoint on each of the coagulation tests is preferable at our present state of knowledge. This will give us the truer physiological picture of clotting in space. The fibrin endpoint may be determined, usually, by photometer [12,13] or by change in electrical impedance. [16] A capillary method would probably be the easiest testing device, however it currently lacks accuracy and reproducibility. Visually observing the fibrin endpoint in a test tube or on a glass slide would rate next on the preferred list.

The additional requirement of a spectrophotometer makes the turbidimetric method somewhat less desirable yet adds little difficulty to the tests. Electrical impedance is probably not feasible since special equipment may well be required. Finally the correlation of esterase (enzymatic) activity [14] with coagulation activity has shown promise; however, we sacrifice the study of coagulation physiology by this method. It is worthy of future consideration. The use of plasma, though preferable to whole blood, is probably not required. If the blood were collected directly into a tubing or vial containing the active reagents, no anticoagulant would be necessary. Therefore, there is considerable flexibility in type specimen that may be used. Most of these tests can be done by direct vision, but are adaptable to turbidimetric methods.

These tests can be done in capillary tubes and only very small volumes of plasma or whole blood are required (0. 1 ml to 0. 2 ml per test).

The space environment introduces some problems in the study of coagulation, as in most other experiments. Careful design of appa-

Coagulation Factors

Merit Parameters	Present Methods					
	Visual Endpoint			Photometer Turbidimetric (Ref 9,10,11 12,13)	Electrical Impedance (Ref 16)	Esterase Activity (Ref 14,15)
	Mix in Test Tube (Ref 1-6)	Mix in Capillary Tube (Ref 1-6)	Mix on Slide or Watch Glass (Ref 7-8)			
1. Sensitivity	10	5	10	10	8	5
2. Sample size	.05 to 0.1 ml	.05 to 0.1 ml	.05 to 0.1 ml	0.3 ml	0.1 ml	0.3 ml
3. Time required	<10 min	<10 min	<10 min	1-30 min	1-30 min	1-30 min
4. Reproducibility	8	5	8	8	8	8
5. Suitability for null gravity use	3	8	5	7	7	7
6. Overall safety	-	-	-	-	-	-
7. Nontoxic reagents	10	10	10	10	10	8
8. Specificity	10	10	10	8	6	5
9. Insensitive to environmental changes	3	3	3	3	3	3
10. Ease in training personnel	6	6	6	8	8	3
11. Degree of separation required (10=none)	8	10	8	8	8	8
12. Minimal handling by analyst	8	10	8	8	8	6
13. Common use of analytic equipment	10	10	10	10	0	8
14. Nondestructive of sample	10	10	10	5	5	5
15. Merit range	21-42	15-31	25-50	26-52	23-46	19-38
16. Mean figure of merit	32	23	38	39	35	29

ratus with consideration of the following items will permit the experiments to be carried out in an orbiting laboratory.

a. Temperature requirements

Most of the reagents used are thermolabile and will require refrigeration or some method of preservation that will protect against loss of activity. The coagulation tests should all be carried out at a controlled fixed temperature.

b. Mixing reagents uniformly and rapidly is crucial and may be difficult in a null gravity environment.

c. Lability of reagents and reactants exposed to ionizing radiation.

d. Variability in collection.

The problems of collection and mixing require special consideration. The following suggestions might provide workable solutions to these problems.

(1) Plasma or blood could be collected directly into capillary tubes containing all the necessary reagents in a liquid or lyophyllized form. Preferably these reagents should be adherent to the inner wall of the capillary tube. Blood would solubilize the reagents, and the ends of the tube then sealed. The endpoint of the reaction would be the formation of a fibrin thread observed by successively breaking the tube at fixed time intervals.

(2) Capillary tubes could also be graduated and could serve as pipettes if desired.

(3) An alternative method would be to collect plasma or whole blood directly into a syringe containing the active reagents. The reagents could be in a liquid or lyophyllized form. Liquid form would be preferable for rapid mixing. Periodic attempts to express blood from the syringe might serve as the measurement. Clotted blood would be more difficult to express from the syringe.

(4) Reagents could be drawn into one end of a Y tube and plasma

the other end. When the reagents meet at the Y junction they would mix. Fibrin formation would be observed at this junction and beyond, as shown in Figure 2.
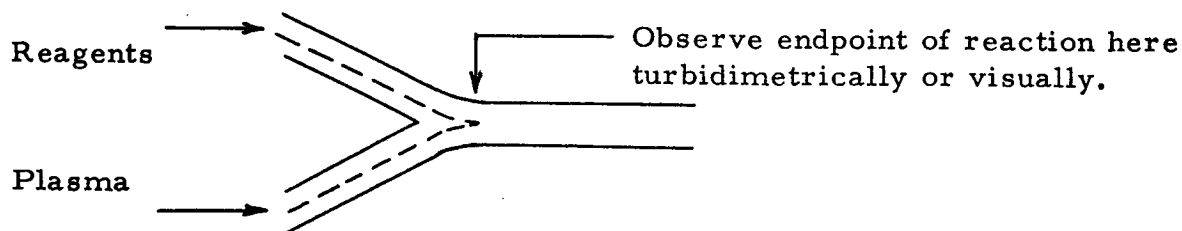

Figure 2. Y-tube for observing fibrin formation.

Finally, attempts to simulate the temperature changes, radiation effects, [20, 21, 22, 23, 24] gravitational alterations, or other variables encountered in the space environment which might handicap proposed methods of study and behavior of reactants should indicate what modifications need to be made. Normal values for the tests might also be established at this time.

Visual methods or spectrophotometric measurements of coagulation are the best approaches for the next two or three years.

D. Areas for research and development

The biochemical study of coagulation shows great potential. The ability of most clotting factors to exhibit enzymatic activity has been demonstrated. The development of specific substrates to measure individual factor activity would seem to be a desirable goal. Quantitation might then be more feasible than it is presently by our grosser, more qualitative measurements. It might also be possible to impregnate porous paper sticks with the active reagents and thus have a dip-stick of measuring the factors possessing enzymatic activity. This may be accomplished in 4 - 6 years with large scale investigations necessary.

Of course, specific molecular identification of these factors may lead to other methods of identification i.e. phospholipid and amino analysis of the clot accelerating lipoproteins. An estimated 7 years or longer will

elapse before this methodology can be developed.

E. References

1. Miale; Laboratory Medicine-Hematology, C.V. Mosby Co., pp. 853 - 885.

   Methodology - Standard test tube methods of detecting clotting abnormalities are described.

   1. Venous coagulation time
   2. Clot retraction
   3. Capillary bleeding time
   4. Plasma recalcification time
   5. One stage prothrombin time (macro & micro)
   6. Partial thromboplastin time (macro & micro)
   7. Thromboplastin generation test
   8. Fibrinolysin
      a. Euglobulin lysis time
      b. Mixture of normal plasma and pot plasma lysis time.

2. Wintrobe; Clinical Hematology, Lea and Febiger, pp. 298 - 307.

   Similar methods to reference 1 are described.

3. Dameshek and Stefanini; The Hemorrhagic Disorders, Grune and Stratton.

   Similar methods as in reference 1.

4. Hougie; Fundamentals of Blood Coagulation in Clinical Medicine, Blakiston-McGraw.

   Similar methods as in reference 1.

5. Biggs, McFarlane; Human Blood Coagulation and its Disorders, Blackwell Publishing Co., pp. 380 - 421.

   Similar methods are described as in reference 1 with some modifications in technique.

6. Quick; Hemorrhagic Diseases and Thrombosis, Lea and Febiger, pp. 384 - 433.

   Similar methods are described as in reference 1 but much less detail given.

7. Cole; Unpublished Results on the Pt. Time Using a Glass Slide.

Uniform heating on a hot plate held at 37° of a glass slide enables coagulation tests to be done by mixing droplets of patients' plasma with reagents in micro quantities. The fibrin endpoint is very easy to see and reproducible results are obtainable by the neophyte.

8. Ulin, A. W.; Gollub, S.; New Rapid, and Practical Micromethods for Prothrombin Determination. J. Lab. Clin. Med., Vol. 50, 1957, pp. 323 - 325.

Method is described whereby a watch glass is used instead of a glass slide as above. No control of temperature is attempted with this method.

9. Lein, J.; A Photometric Analysis of the Reactions of Blood Coagulation. J. Cellular Comp. Physiol., Vol. 30, 1947, pp. 43 - 77.

Method is described by which light scattering can be measured in the thrombin-fibrinogen interaction. Possibility is that this method could be used to titrate fibrinogen content.

10. Harrower, H. W.; Brook, D. L.; An Instrument for the Study of Blood Coagulation and Lysis. Ann. Surg., Vol. 160 (5), 1964, pp. 870 - 883.

A modified photometer is described for the study of blood coagulation (on citrated whole blood, plasma, and citrated plasma) with the end point automatically recorded.

11. Gruendlinger, J.; Bouvier, C. A.; Applications of a Photometric Method to the Study of Coagulation and Fibrinolysis Phenomena. Thromb. Diath. Haemorrhag., Vol. 12 (3-4), 1964, pp. 425 - 451.

A method whereby fibrinolysis can be measured using the spectrophotometer.

12. Stefanini, M.; The Significance of the Photometric Method for the Study of the Blood-Coagulation Process. Policlinico, (Rome), Vol. 48, 1941, pp. 313-323.

Describes four stages of coagulation measured by changes in optical density which reflect the changes and abnormalities of the coagulation process.

13. Bouviow, C. A.; Grundlinger, J.; Investigations on Coagulation and Fibrinolysis Phenomena by a Spectrophotometric Method of Continuous Recording. Schweiz. Med. Wochschr., Vol. 93, 1963, pp. 1451 - 1455.

A method of measuring fibrinolysis using the spectrophotometer is described.

14. Hough, D. A.; Lyons, L. V.; Koppel, J. L.; Olwin, J. H.; Spectro-photometric Modification of the TAMe (p-tosyl-L-arginine methyl ester) Assay for Prothrombin. Use for Control of Anticoagulant Therapy. J. Am. Med. Assoc., Vol. 188 (3), 1964, pp. 207 - 211.

Method described in this paper measures the esterase activity generated in the act of clotting as determined by the esterolytic action on TAMe (p-tosyl-L-arginine methyl ester). This is measured spectrophotometrically.

15. Glueck, H. I.; The Utilization of Synthetic Substrate (p-toluene-sulfoyl-L-argine methyl ester) to Measure the Plasma Prothrombin in Coagulation Disorders. J. Lab. Clin. Med., Vol. 49, 1957, pp. 41 - 60.

Similar method as above but measuring only Stage II of clotting.

16. Bayo Bayo, J. M.; Eder Zuluanga Jaramillo; Method of Study of Blood Coagulation Based on the Variation in Electric Resistance of the Fibrin Net. Arch. Inst. Farmacol. Exptl. (Madrid), Vol. 15 (1), 1963, pp. 75 - 93.

Method described to detect the fibrin endpoint of coagulation by electrical impedance generated in a plasma solar, as gel formation occurs. This could be used to measure fibrinogen concentration and perhaps other factors.

17. Caen; Michel, H.; Aggregation of Platelets with ADP. Rev. Franc. Etudes Clin. Biol., Vol. 9 (9), 1964, pp. 999 - 1000.

Visual method is described whereby platelets are aggregated by ADP.

18. Vainer, H.; Caen, J.; A Photometric Test for Studying the Effect of Adenosine Diphosphate (ADP) on the Blood Platelets. Nouvelle Rev. Franc. Hematol., Vol. 3, 1963, pp. 149 - 157.

Spectrophotometric measurement of the effect of ADP on platelet aggregation.

19. Born, G. V. R.; Cross, M. J.; Aggregation of Blood Platelets. J. Physiol., (London), Vol. 168 (1), 1963, pp. 178 - 195.

The use of platelet aggregation as a method of determining platelet concentration. The degree of platelet aggregation by ADP is proportional to the concentration of platelets present in the plasma. This can be done turbidimetrically.

20. Vasilevskii, N. M.; Blood Clotting Following Exposure to Ionizing Radiation. Zdravookhranenie Belorussii, Vol. 1, 1957, pp. 136 - 137.

21. Verwilghen, R. L.; J. M. Peremans; Changes in the Prothrombin Complex After Total-Body Irradiation. U.S. Armed Forces Med. J., Vol. 6, 1955, pp. 645 - 656.

22. Stender, H. S.; Elbert, O.; Effect Upon Blood Coagulation Factor After Whole Body Irradiation With Large Doses of Radiation. Strahlentherapie, Vol. 90, 1953, pp. 625 - 632.

23. Ponomarev. Yu. T.; Dynamics of Changes in Coagulative and Fibrinolytic System of Dog Blood in Acute Radiation Sickness. Radiobiolgiya, Russian, Vol. 5 (4), 1965, pp. 519 - 521.

24. Savitsky, J. P.; Sherry, S.; Changes in Clot Retraction Time and Platelet Adhesiveness Following Whole-Body Irradiation. Proc. Soc. Exptl. Biol. Med., Vol. 85, 1954, pp. 587 - 590.

References 20 - 24: Describes the changes in blood clotting induced by varying degrees of radiation exposure in experimental animals.

3.3.04 <u>Fibrinogen (Plasma)</u>

  A. Principles of present methodology

      1. Martinek[4]

Principle: Addition of 1.2 <u>M</u> phosphate buffer to plasma results in the precipitation of fibrinogen. The turbidity is measured at 450 mμ against a blank prepared by adding an equal volume of plasma to 0.9% NaCl solution. The relationship between absorbance values and fibrinogen concentrations is linear. The precipitating reagent is stable at room temperature for at least 2 years. Heparin, sodium citrate or EDTA are suitable as anticoagulants. Hemolysis must be avoided. Cuvettes with a light path greater than 1 cm will increase the sensitivity of the procedure. The instrument is calibrated with fibrinogen solutions of known concentrations. The total volume of plasma required is 0.5 ml but it probably can be reduced to 0.2 ml.

      2. Ellis and Stransky[1]

Principle: Addition of a calcium-thrombin solution to plasma converts fibrinogen to fibrin. The degree of turbidity is directly related to the fibrinogen content of plasma.

Procedure: Plasma is diluted with 0.1 <u>M</u> barbital buffer. The solution is divided into two equal parts. One aliquot is used as the "blank" and the other as the "test". One drop of calcium-thrombin solution is added to the "test" and is allowed to clot for 20 minutes. The absorbance of the "test" is measured against the blank at 470 mμ in a spectrophotometer. The instrument is calibrated with fibrinogen solutions of known concentrations (calibration curve). A straight line is obtained when absorbance values are plotted versus fibrinogen concentrations.

Citrate is the only suitable anticoagulant. The calcium-thrombin solution is stable for at least 3 months at -20° C. Bilirubin, lipemia and moderate hemolysis do not interfere with the determination.

3. Rafferty et al.[5]

Principle: Fibrinogen is precipitated from plasma by the addition of ammonium sulfate. The resulting turbidity is measured in a nephelometer. Nephelometric techniques provide better sensitivity in measuring low concentrations of particles which are beyond the sensitivity of turbidimetric measurements. With this method it is possible to reduce the volumes of plasma required for the analysis of fibrinogen to approximately 0.05 ml.

B. Suitability of present methodology to space flight conditions

1. Merit table

Fibrinogen in Plasma

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Fair	Fair	Good
2. Sample size	0.5 ml	0.5 ml	.1 ml
3. Time required	3 min	25 min	5 min
4. Reproducibility	8	9	-
5. Suitability for null gravity use	9	8	9
6. Overall safety	10	9	10
7. Nontoxic reagents	10	8	10
8. Specificity	8	9	8
9. Insensitive to environmental changes	10	3	9
10. Ease in training personnel	10	6	9
11. Degree of separation required (10=none)	9	9	9
12. Minimal handling by analyst	9	5	8
13. Common use of analytic equipment	10	10	7
14. Noncaustic reagents	10	10	10
15. Nondestructive of sample	1	1	1
16. Merit range	-	-	0-83
17. Mean figure of merit	70	57	42

2. Discussion

Methods 1, 2, and 3 are turbidimetric, and a difficult aspect of

turbidimetry or nephelometry is the preparation of reproducible and stable suspensions. The rate of addition of reagent, the manner of mixing, and shaking and other variables must be kept under control.

Method 2 probably is more specific and should give more accurate results. Fibrinolysis is a potential source of error with this method as it may occur before the turbidity of the clot is measure; it can be avoided by allowing the plasma to stand at room temperature for several hours.

Numerous precipitating reagents, i. e. , sodium sulfite, sodium sulfate, sodium chloride and heat, have been used for the measurement of fibrinogen. However, they do not offer any significant advantage over those already described.

Choice: The Method of Martinek should be the method of choice at the present. It is simple, rapid and requires a small volume of plasma.

<div align="center">Equipment</div>

Method 1: Spectrophotometer and centrifuge

Method 2: Spectrophotometer and centrifuge

Method 3: Nephelometer and centrifuge

C. Areas for research and development

1. The nephelometric method should be explored. The available data with regard to its precision and standardization are meager. Its sensitivity is a very desirable feature.

2. Attempts have been made to determine fibrinogen by differential refractometry.[3] The difference between the refractive index of plasma and serum can serve as a means for estimating the fibrinogen concentration. However, the sensitivity, reproducibility, accuracy and normal values have not been reported.

D. References

1. Ellis, B. C. ; Stransky, A. ; A Quick and Accurate Method for the Determination of Fibrinogen in Plasma, J. Lab. Clin. Med. , Vol. 58, 1961, pp. 477 - 488.

Fibrinogen is measured by the turbidity produced when it is polymerized to fibrin by thrombin.

2. Gleye, M.; Préparation et Utilisation d'un Immunsérum Spécifique du Fibrinogène. Compt. Rend., 1962, pp. 2685 - 2686.

Describes the preparation of an antiserum specific for human fibrinogen.

3. Leendertz, G. Eine Klinische Methode Zur Bestimmung des Blutfibrinogens. Klin. Wochschr., Vol. 2, 1963, pp. 746 - 747.

The difference in the refractive index between plasma and serum can be used for the estimation of fibrinogen.

4. Martinek, R.G.; Berry, R.E.; Micromethod for the Estimation of Plasma Fibrinogen. Clin. Chem., Vol. 11, 1965, pp. 10 - 16.

Fibrinogen is determined turbidimetrically after it is flocculated with 1.2 $\underline{M}$ phosphate buffer.

5. Rafferty, N.S.; Tyrol, A.G.; Parfentjev, I.A.; Turbidimetric Analysis of Fibrinogen and Gamma Globulin with Ammonium Sulfate by Different Photometric Technics. Clin. Chem., Vol. 4, 1958, pp. 185 - 193.

Fibrinogen is measured with a nephelometer after it is flocculated with $(NH_4)_2 \ SO_4$.

3.3.05 Hemoglobin

I. In blood

   A. Principles of present methodology

      1. Oxyhemoglobin

There are numerous modifications of the oxyhemoglobin method but the procedure proposed by Collier[2] is the most attractive.

Principle: Whole blood is diluted and hemolyzed with a 0.3% solution of the tetrasodium salt of ethylenediaminetetraacetic acid (EDTA-Na$_4$) and the hemoglobin is fully oxygenated by exposure to air. The oxyhemoglobin is determined from its absorbance at 540 mμ.

Oxyhemoglobin standards are not available; this method should be standardized against the cyanmethemoglobin method or by determination of iron.

The only reagent required is EDTA-Na$_4$ which is stable and nontoxic.

The oxyhemoglobin color develops in a few seconds (the oxygenation of hemoglobin is very rapid) and stable for at least 16 hours.

In the absence of lipemia, the accuracy of the techniques is limited by the absence of abnormal hemoglobin pigments; methemoglobin and carboxyhemoglobin are not converted to oxyhemoglobin.

Due to the presence of EDTA the accuracy of the method is not affected by the presence of copper ions.

      2. Cyanmethemoglobin[4]

Principle: Whole blood is diluted with a potassium ferricyanide-potassium cyanide (K$_3$Fe(CN)$_6$-KCN) solution that oxidizes hemoglobin to methemoglobin which is, in turn, converted to cyanmethemoglobin. The absorbance of cyanmethemoglobin is measured at 540 mμ. This procedure will measure hemoglobin, oxyhemoglobin, methemoglobin, and carboxyhemoglobin.

The Panel on the Establishment of a Hemoglobin Standard of the

of Medical Sciences, National Academy of Sciences, National Research Council has recommended universal adoption of the cyanmethemoglobin method because of its good accuracy and precision.

However, the presence of potassium cyanide is potentially hazardous to the health of the astronauts.

Cyanmethemoglobin standards are commercially available and very stable under refrigeration.

3. Azide methemoglobin

Vanzetti[5] proposed replacement of the toxic KCN with the much less toxic sodium azide.

Principle: Whole blood is diluted with potassium ferricyanide-sodium azide $(K_3Fe(CN)_6-NaN_3)$ solution which converts hemoglobin to azide-methemoglobin. The absorbance of azide-methemoglobin is measured at 540 mμ. The method has not gained wide acceptance but the author claims that its accuracy and precision are identical with those of cyanmethemoglobin.

The absorption coefficients of cyanmethemoglobin and azide-methemoglobin are identical at 540 to 546 mμ but the absorption spectra of the two hemoglobin derivatives are not. A plateau is seen in the azide-methemoglobin curve at 575 mμ.

B. Suitability of present methodology to space flight conditions

1. Merit table

Hemoglobin in Blood

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	.2 g	.2 g	.2 g
2. Sample size	20 μl	20 μl	20 μl
3. Time required	1 min	20 min	10 min
4. Reproducibility	9	9	9
5. Suitability for null gravity use	9	9	9
6. Overall safety	9	1	5
7. Nontoxic reagents	10	1	5

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Hemoglobin in Blood - continued

Merit Parameters	Method 1	Method 2	Method 3
8. Noncaustic reagents	9	9	9
9. Specificity	9	9	9
10. Insensitive to environmental changes	10	2	2
11. Ease in training personnel	10	10	10
12. Degree of separation required (10=none)	10	10	10
13. Minimal handling by analyst	9	9	9
14. Common use of analytic equipment	10	10	8
15. Nondestructive of sample	0	0	0
16. Figure of merit	**74**	**30**	**46**

2. Discussion

It seems that the method of choice for the determination of hemoglobin is the oxyhemoglobin method.

The only reagent needed is EDTA-Na$_4$ which is stable even in solution for several months at room temperature. This compound has the advantage over the KCN and NaN$_3$ that it is nontoxic and therefore safe to use. The oxyhemoglobin method is more rapid than the other two. The conversion of hemoglobin to oxyhemoglobin in the presence of air is almost instantaneous.

It has been argued that the oxyhemoglobin method does not measure any methemoglobin and/or carboxyhemoglobin present in the blood. However, both methemoglobin and carboxyhemoglobin absorb light at 540 mμ and the error introduced by significant quantities of methemoglobin and carboxyhemoglobin is no more than a few percent.[1]

Sodium azide is a known metabolic inhibitor and ingestion of the compound will cause hypotension. The fatal dose of NaN$_3$ for humans remains unknown. Studies on laboratory animals have indicated that it is 6 - 7 times less toxic than KCN.

Equipment

Oxyhemoglobin Method: Spectrophotometer or filter
colorimeter

Cyanmethemoglobin Method: Spectrophotometer or
colorimeter

Azide-methemoglobin Method: Spectrophotometer or
colorimeter with narrow
band width filter.

The present methods for the estimation of blood hemoglobin
are precise, simple and require minimal handling. Only dilution
of blood is necessary and that can be easily accomplished with an
automatic diluting pipette. There is a possibility of measuring
hemoglobin in undiluted blood samples by means of a cuvette with
a very short light path. Such cuvettes with a light path of 0.07 mm
have been constructed by Drabkin and Austin[3] but the accuracy and
precision of their method was never evaluated.

C. Areas for research and development

(None)

D. References

1. Bell, G.H., et al.; The Routine Estimation of Haemoglobin as
Oxyhaemoglobin. Biochem. J., Vol. 39, 1945, pp. 60 - 63.

   At 520 mμ all the hemoglobin pigments likely to be found in the
blood had approximately the same extinction coefficient.

2. Collier, H.B.; The Use of Sequestering Agent in Determination of
Oxyhemoglobin. Am. J. Clin. Path., Vol. 25, 1955, pp. 221 -
222.

   Suggests the replacement of dilute NH_4OH with tetrasodium
versenate solution in the oxyhemoglobin method.

3. Drabkin, D.L.; Austin, J.H.; A Technique for the Analysis of Un-
diluted Blood and Concentrated Hemoglobin Solution. J. Biol.
Chem., Vol. 112, 1935, pp. 105 - 115.

   Describes spectrophotometry of hemoglobin in undiluted blood
using a cuvette with narrow light path.

4. Hainline, A., Jr.; Hemoglobin, Standard Methods of Clinical Chemistry. Vol. 2, ed. D. Seligson, Academic Press, N.Y., 1958, pp. 49 - 60.

5. Vanzetti, G.; An Azide-methemoglobin Method for Hemoglobin Determination in Blood. J. Lab. Clin. Med., Vol. 67, 1966, pp. 116 - 126.

   Describes the estimation of blood hemoglobin by conversion to azide-methemoglobin and spectrophotometric measurement at 542 mμ.

II. In plasma

The more sensitive method for the determination of plasma hemo-globin was introduced by Hanks et al.[2] and it is a modification of the method of Crosby and Furth.[1] Both methods are chemical.

A direct spectrophotometric method has been described by Martinek[3] which requires only 0.9% NaCl as reagent.

A. Principles of present methodology

1. Hanks et al.[2]

Principle: Hemoglobin catalyzes the oxidation of benzidine to a green product by oxygen released from hydrogen peroxide.

Procedure: Plasma is mixed with a solution of benzidine in glacial acetic acid. Hydrogen peroxide is added and the absorbance of the resulting color solution is measured after 3-1/2 minutes at 700 mμ.

The method can measure amounts of hemoglobin from 0 to 4 mg per 100 ml of plasma.

The time for color development is very critical and the benzedine solution must be used within 6 hours after its preparation.

The normal values for this procedure are the lowest ever reported; mean value 0.32 mg%(0.16 - 0.58 mg%.)

2. Crosby and Furth[1]

Principle: Same as in Method 1.

Procedure: Plasma is mixed with a benzidine solution in 90% acetic acid. Hydrogen peroxide is added and the mixture is incubated for 20 minutes at room temperature. It is diluted with 10% acetic acid and the absorbance is measured at 515 mμ. The precision of the method is poor when the hemoglobin level is below 4 mg%.

3. Martinek[3]

Principle: The absorbance of serum or plasma diluted with saline is measured at 412 - 415 mμ (Soret peak of oxyhemoglobin).

The spectrophotometer is calibrated with oxyhemoglobin solution of known concentrations. A correction is made for the interference caused by bilirubin by measuring the optical density of the diluted plasma at the peak of the bilirubin absorption, 455 - 457 mμ. The correction is based on the fact that the absorbance of bilirubin at 412 - 415 mμ is 80% of its peak absorbance. This method is very recent and has not been evaluated. However, the author claims that the reliability of the method has been established by comparison with the methods of Hanks, et al.[2]

Fasting blood specimens are mandatory with this procedure.

B. Suitability of present methodology to space flight conditions

    1. Merit table

Hemoglobin in Plasma

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Excellent	Good	-
2. Sample size	0.4 ml	0.02 ml	0.1 ml
3. Time required	5 min	35 min	2 min
4. Reproducibility	8	8	-
5. Suitability for null gravity use	5	5	8
6. Overall safety	1	1	10
7. Nontoxic reagents	3	3	10
8. Noncaustic reagents	1	1	10
9. Specificity	9	9	8
10. Insensitive to environmental changes	0	3	10
11. Ease in training personnel	2	6	9
12. Degree of separation required (10=none)	9	9	9
13. Minimal handling by analyst	3	5	9
14. Common use of analytic equipment	10	10	10
15. Nondestructive of sample	1	1	8
16. Merit range	-	-	0-84
17. Mean figure of merit	13	15	42

2. Discussion

Benzidine has been reported to cause carcinoma of the bladder. Methods 1 and 2 require the use of glacial acetic acid which is highly undesirable. Therefore, they cannot be recommended.

A spectrophotometer and a centrifuge are required for all of the described methods.

C. Areas for research and development

Method 3 is very promising and should be evaluated. If reliable it will undoubtedly be the method of choice.

D. References

1. Crosby, W.H.; Furth, F.W.; A Modification of the Benzidine Method for Measurement of Hemoglobin in Plasma and Urine. Blood, Vol. 11, 1956, pp. 380 - 383.

    Procedure for measuring hemoglobin levels in plasma above 4 mg%.

2. Hanks, G.E.; Casseli, M.; Ray, R.N.; Chaplin, H., Jr.; Further Modification of the Benzidine Method for Measurement of Hemoglobin in Plasma. J. Lab. Clin. Med., Vol. 56, 1960, pp. 486 - 498.

    Describes a highly sensitive method capable of measuring amounts of hemoglobin from 0 to 4 mg per 100 ml of plasma.

3. Martinek, R.G.; A Simple Spectrophotometric Method for Quantitating Hemoglobin in Serum or Plasma. J. Am. Med. Technol., Vol. 28, 1966, pp. 42 - 58.

    The method utilizes the absorbance of oxyhemoglobin at the Soret band, 412 - 415 mμ.

III. In Urine

One has to distinguish between hematuria and hemoglobinuria. The first term indicates the presence of an abnormal number of red blood cells in the urine. The second term refers to the presence in the urine of hemoglobin or its derivatives in solution.

Hematuria can be detected by microscopic examination of the sediment following centrifugation of a urine aliquot. According to Cook et al[3]. the number of erythrocytes must be close to 50 million per liter of urine before the presence of an abnormal content can be detected with certainty. The sensitivity of the microscopic examination will decrease with hemolysis of the erythrocytes in the urine. Adams et al.[1], and Leonards[5], reported that in over 95% of the urines containing blood some of the blood was hemolyzed. The extent of hemolysis may range from a small proportion of the total blood excreted to the extreme where all the excreted blood is hemolyzed. Therefore, microscopic examination of the urinary sediment cannot always give a reliable indication of the total amount of hemoglobin.

Methods for the detection of occult blood in urine must be able to detect both intact red blood cells and hemoglobin in solution. Reagents such as benzidine, guaiac and o-tolidine have been employed for the detection of occult blood in urine. The most sensitive of the three is o-tolidine.

A. Principles of present methodology

    1. Stewart and Dunlop[6]

        Principle: Hemoglobin and its derivatives catalyze the oxidation of o-tolidine to a blue colored product by oxygen released from hydrogen peroxide.

        Specimen: Uncentrifuged urine heated to boiling point and cooled.

        To a mixture of o-tolidine solution and hydrogen peroxide a few drops of boiled urine are added. In the presence of hemoglobin a blue color appears in approximately one minute.

The sensitivity of the test varies between samples because of the presence of inhibitors in urine.

True peroxidases which may be present in urine are destroyed by boiling the urine.

The stock o-tolidine solution (4 g in 100 ml of ethanol) is stable for a long time at 4° C.

The sensitivity of the test is decreased by high normal levels of ascorbic acid in urine and drastically decreased by therapeutically high levels.[3]

2. "Occultest"[*]

"Occultest" is a tablet containing o-tolidine, strontium peroxide, calcium acetate, sodium bicarbonate and tartaric acid and is manufactured by Ames Company.

The test for occult blood is performed as follows: One drop of uncentrifuged urine is placed in the center of the piece of a filter paper and an "Occultest" tablet is applied on the top of the moist area. Two drops of water are placed on the tablet. The appearance of a blue color on the paper within 2 minutes indicates the presence of blood. This test is very sensitive and in most cases a positive reaction is obtained with "Occultest" when one part of blood is present in 300,000 parts of urine.[3]

Free et al.[4], found good correlation between the results with the "Occultest" on uncentrifuged urine and those obtained by microscopic examination of the urine sediment.

3. "Hema-Combistix"[*] (dip stick)

The "Hema-Combistix" is a paper strip with four areas, one of which is used for the detection of occult blood in urine; the other three areas are used for the detection of glucose, protein and determination of urinary pH. The area used for the detection of occult blood is impregnated with o-tolidine, a peroxide and suitable buffers. A blue color appears in the area for the detection of

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[*] Registered trademark of the Ames Co., Inc., Elkhart, Indiana.

blood when the dip stick is immersed in a urine specimen containing blood. The usefulness of the "Hema-Combistix" in the detection of occult blood in urine has been evaluated by several investigators.[2, 7] In general good correlation was found with the results of "Hema-Combistix" and those obtained by microscopic examination of the urinary sediment. However, in a few cases the dipstick test was negative while there were from 5 to 14 red blood cells per high-power field. In some of these cases the "Hema-Combistix" was inhibited by a high ascorbic acid concentration in the urine.

B. Suitability of present methodology to space flight conditions

   1. Merit table

Hemoglobin in Urine

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Excellent	Very Good	Very Good
2. Sample size	1 ml	.05 ml	.10 ml
3. Time required	5 min	1 min	1 min
4. Reproducibility	9	9	9
5. Suitability for null gravity	6	10	10
6. Overall safety	1	10	10
7. Nontoxic reagents	1	10	10
8. Noncaustic reagents	1	9	10
9. Specificity	8	8	8
10. Insensitive to environmental changes	2	8	10
11. Ease in training personnel	8	10	10
12. Degree of separation required (10=none)	10	10	10
13. Minimal handling by analyst	3	6	10
14. Common use of analytic equipment	3	7	10
15. Nondestructive of sample	0	2	10
16. Figure of merit	15	76	87

2. Discussion

The o-tolidine test is undoubtedly the most sensitive for the detection of occult blood in urine. However the test requires reagents which may be undersirable and dangerous, i.e., glacial acetic acid, ethanol, hydrogen peroxide; therefore, the "Occultest" and "Hema-Combistix" are much more suitable for urine hemoglobin.

Although the sensitivity of "Hema-Combistix" is inferior to that of the "Occultest", the former is preferable because of its simplicity and speed.

C. Areas for research and development

Efforts would be made to prepare a dip stick with greater sensitivity.

Equipment

Method 1:	Heater
Method 2	"Occultest" tablets
Method 3:	"Hema-Combistix" strips

D. References

1. Adams, E. C., Jr.; Fetter, M. C.; Free, H. M.; Free, A. H.; Hemolysis in Hematuria. J. Urol., Vol. 88, 1962, pp. 427 - 430.

    Urine specimens containing occult blood were found to have hemolyzed blood in 90% of the cases.

2. Budinger, J. M.; Cavallo, M.; Detection of Hematuria with a Paper Strip Indicator. Am. J. Clin. Path., Vol. 42, 1964, pp, 626 - 629.

    The "Hema-Combistix" gives a positive test when the number of erythrocytes per high-power field is approximately 3.

3. Cook, M. H., et al.; The Detection of Blood in Urine. Am. J. Med. Tech., Vol. 22, 1956, pp. 218 - 231.

    Various methods of detecting blood in urine are compared with regard to sensitivity and accuracy.

Test for the Detection of Occult Blood in Urine. J. Urol., Vol. 75, 1956, pp. 743 - 752.

The "Occultest" is a simple and an accurate test for occult blood in urine.

5. Leonards, J.R.; Simple Test for Hematuria Compared with Established Tests., J.A.M.A., Vol. 179, 1962, pp. 807 - 808.

Hemolysis occurs in 95% of the cases in urines containing occult blood.

6. Stewart, C.P.; Dunlop, D.M.; Clinical Chemistry in Practical Medicine, Livingstone, London, 1958, pp. 287 - 305.

Application of the o-tolidine test for the detection of hemaglobin in urine.

7. Yoder, J.M.; Adams, E.C.; Free, H.M.; Simultaneous Screening for Urinary Occult Blood, Protein, Glucose, and pH. Am. J. Med. Tech., Vol. 31, 1965, pp. 285 - 290.

Good correlation exists between the results with "Hema-Combistix" and those obtained with microscopic examination of the urine sediment.

3.3.06 <u>Immune bodies</u>

This discussion will concentrate on the study of serum proteins, some of which have immunological activity. The laboratory procedures principally evaluated for this project include zone electrophoresis, immunoelectrophoresis and immunodiffusion.

I.   Electrophoretic analysis

Electrophoretic analysis affords determination of a broader spectrum of serum proteins. Other chemical methods of serum albumin and globulin fractionation are not so sensitive as electrophoretic separation. Any changes observed with one or more of the five protein fractions obtained with zone electrophoresis can be a sensitive indicator of pathophysiologic changes.

A. Principles of present methodology

The physical principle underlying zone electrophoresis is stated as follows: particles carrying an electric charge are accelerated when placed in an electric field. The particles move at a constant speed proportional to their charge. This driving force is balanced by frictional forces arising in the medium.[10] These forces operate regardless of the type of medium (agar gel, starch gel, cellulose acetate) used.

B. Suitability of present methodology to space flight conditions

1. Merit table

<div align="center">Immune bodies</div>

Merit Parameters	Agar Gel	Cellulose Acetate	Starch Gel (micro-methods)
1. Accuracy[*]	$\pm$ 25%	$\pm$ 4 to $\pm$ 15%	**
2. Sample size	0.006 ml	0.01 to 1.0 μl	0.015 ml
3. Time required	2 hrs.	approx. 1 hr.	2 hrs.
4. Reproducibility	8	8	7
5. Suitability for null gravity use	5	5	4

Immune bodies - continued

Merit Parameters	Agar Gel	Cellulose Acetate	Starch Gel (micro-methods)
6. Overall safety	4	4	4
7. Nontoxic, noncaustic reagents	2	2	2
8. Specificity	10	10	10
9. Insensitive to environmental change	8	8	8
10. Ease in training personnel	10	10	8
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	8	10	7
13. Common use of analytic equipment	3	3	3
14. Nondestructive of sample	0	0	0
15. Figure of merit	28	29	22

*   This includes **sensitivity** for all of the separated fractions.

**   **It is difficult to report on the accuracy of this procedure since quantitation of these gels has not been very practical or definitive.**

2. Discussion

The technique of choice for zone electrophoresis, for the primary investigations in this project, is the micromethod described by Grunbaum and Durum.[3, 4] This method makes use of cellulose acetate strips in a micro-electrophoresis apparatus. The Microzone Electrophoresis Cell (Model R - 101)[1] can be easily adapted and is available commercially.[+] After electrophoresis is completed, the strips have to be stained for the localization and identification of the protein fractions. Such stains as Amido-Black 10B, Bromphenol Blue and Ponceau S can be used. The separated protein fractions can be quantitated on a photodensitometer scanner-recorder. If desired, this can be done post-flight since the stained strips can be kept for a long period of time.

Microzone cellulose acetate electrophoresis has the shortest electrophoresis time (20 minutes) of the three methods compared

[+] Beckman Instruments, Inc., Standard Industrial Park, Palo Alto, California.

here.

The rapid micromethods of starch gel electrophoresis are evaluated here. The starch gel for this purpose can be molded on either glass slides or in plastic trays.[6, 7] The electrophoresis time for these modified procedures can be reduced to about two hours or less. This is an advantage over the original method described by Smithies[8] which required 18 to 22 hours of electrophoresis time. One major drawback in the use of these gels is that they cannot be prepared in advance of the experiment. Personal experience as well as statements made in the literature[9] indicates that it is not feasible to store these gels for periods longer than one or two days at refrigerator temperatures. Consequently, these gels will have to be prepared during flight if the investigative period will extend into several days.

Electrical contact with gels or cellulose acetate strips is usually achieved by their direct immersion into compartments filled with a selected buffer solution. Major modifications may have to be made here for adapting these procedures to space flight. An ultramicromethod of electrophoresis described by Fischl and Gordon[2] offers several promising modifications for this project. The electrophoresis cell described is small, 40 x 30 x 12 mm. The electrode compartments are 20 x 40 mm and 4 mm deep. Pieces of cellulose sponge 16 x 4 mm and 3 mm, when dry, are placed into the electrode compartment and 0.35 ml of barbiturate buffer (0.05 M, pH 8.6) is added to both. The optimal conditions are 60 volts for 15 minutes or if very rapid results are required, 120 volts for 5 minutes is possible. Wet or dry storage batteries may be used for power supply. The batteries can be connected to the apparatus by copper foils.

For the initial experiments, zone electrophoresis utilizing cellulose acetate membranes appears to be the best approach.

Micro-methods of starch gel electrophoresis can be adapted at a later date if changes in electrophoretograms are noted in astronauts during flight. One major drawback to the use of these procedures is the staining process which requires the use of glacial acetic acid and methanol.

C. Area for research and development

The use of fluorescence depolarization methods in which the antigen is tagged with a fluorescent dye may permit detection of small amounts of antibody even under conditions where precipitation does not occur. Research in this area may lead to much more precise analysis. This approach may also prove useful in other applications of antigen-antibody reactions. For example it is also possible to employ fluorescent tagged antibody to detect antigens.[5]

D. References

1. Beckman Technical Bulletin, RB-TB-004, Model R-101, Micro-zone Electrophoresis Cell, Preliminary Instruction Manual, 1963.

   This manual describes the commercial adaptation of the micro-electrophoresis setup. A detailed outline of the procedure as used in laboratories is presented.

2. Fischl, J.; Gordon, C.; Ultramicro Electrophoresis. Clin. Chem., Vol. 12, 1966, pp. 887 - 889.

   This article describes an ultramicro-electrophoresis apparatus. Important aspects of this setup are that it makes use of a few drops of buffer and a low voltage.

3. Grunbaum, B.W.; Collins, C.C.; A Self-Contained Microelectro-phoresis Apparatus. Microchem. Journ., Vol. 7, 1963, pp. 283 - 286.

   This paper describes a minature version of the microelectro-phoresis procedure described in reference 4 below.

4. Grunbaum, B.W.; Zec, J.; Durrum, E.I.; Application of an Improved Microelectrophoresis Technique and Immunoelectrophoresis of the Serum Proteins on Cellulose Acetate. Microchem. Journ., Vol. 7, 1963, pp. 41 - 53.

Describes the adaptation of the microelectrophoresis technique and equipment using cellulose acetate for the study of protein fractions. The advantage of cellulose acetate over filter paper and gels is presented.

5. Haber, E.; Bennett, J.C.; Polarization of Fluorescence as a Measure of Antigen-Antibody Interaction, Proc. Nat. Acad. Sci., Vol. 48, 1935, pp.

Detection of antigen-antibody combinations was followed by the decrease in depolarization of fluorescence of fluorescent labeled antibody. This permitted a great increase in sensitivity for detection of soluble antigen-antibody complexes.

6. Marsh, C.J.; Jolleff, C.R.; Payne, L.C.; A Rapid Micromethod for Starch-Gel Electrophoresis. Amer. J. Clin. Path., Vol. 41, 1964, pp. 217 - 223.

A description of a rapid micromethod of starch-gel electrophoresis is presented. The gels are molded in plastic trays 3 mm in depth and 23 cm in length. Actual electrophoresis time is 80 minutes.

7. Ramsey, H.A.; Thin-Layer Starch Gel Electrophoresis on Glass Slides. Analytical Biochem., Vol. 5, 1963, pp. 83 - 91.

The advantages of thin layer starch gel electrophoresis over the use of a thicker gel are presented in this article. In this method the gel is poured as a thin layer on glass slides.

8. Smithies, O.; Zone Electrophoresis in Starch Gel and Its Application to Studies of Serum Proteins. Adv. Protein. Chem., Vol. 14, 1959, pp. 65 - 111.

This is a thorough and concise article that deals with starch gel electrophoresis and its application to studies of serum proteins.

9. Smithies, O.; Zone Electrophoresis in Starch Gels: Group Variations in the Serum Proteins of Normal Human Adults. The Bioch. Journ., Vol. 61, 1955, pp. 629 - 641.

This article deals with the advantages of starch gel electrophoresis such as its greater resolving power and sharper separation. Genetic variation of proteins is presented.

10. Wieme, R.J.; Agar Gel Electrophoresis, Elsevier Publishing Co.,

New York, 1965.

This is a well illustrated textbook discussing the physical aspects of electrophoresis. The applications of agar gel as a suitable electrophoretic medium are also presented.

II. Immunochemical analyses

In this section, two immunochemical methods will be compared, namely, immunoelectrophoresis and immunodiffusion. With either of these two sensitive methods, several specific proteins can be evaluated. Immunoelectrophoresis can be used as a screening procedure since it affords a qualitative measurement of at least 15 - 20 different serum proteins. With immunodiffusion, on the other hand, a quantitative estimation of several specific proteins (such as immunoglobulins, (IgG, IgA, and IgM), transferrin and one of the complement components, β_{1c}) of interest in this project is possible.

A. Principles of present methodology

1. Immunoelectrophoresis

This involves the electrophoretic separation of mixtures of serum proteins in a medium followed by the diffusion of precipitating antibodies in the same medium at right angles to the electrophoresis axis.[6] During the diffusion phase the fractionated proteins react with specific antibodies to produce visible precipitin arcs.

2. Immunodiffusion (Oudin Diffusion Procedure)

The appropriate antiserum (that which contains antibody specific for the protein to be analyzed) is mixed with the buffered agar gel. The serum sample is inoculated on the surface of the solidified agar. The agar can be molded on either petri dishes, glass columns[3] and on 35 mm Cronar polyester photographic film[*] base strips.[1]

Diffusion of the serum sample is allowed to proceed in a moist chamber at room temperature or at 37° C for 18 to 24 hours. The endpoint of this reaction is a precipitation ring formed around the inoculation well when a flat surface is used, or if columns are used, an antigen-antibody front is observed and its distance of migration measured. The diameter of the precipitate is directly related to

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[*] E.I. DuPont de Nemours & Co., 4560 Tuohy Ave., Chicago, Illinois.

the initial concentration of the antigen in solution when temperature and antibody concentration remain constant. The diameter of the precipitation rings of the standards are measured and plotted on semi-logarithmic paper as concentration in mg/100 ml vs. diameter in millimeters. The concentration of the specific protein under analysis is determined by referring to the standard curve.[3]

B. Suitability of present methodology to space flight conditions

    1. Merit table

Immunochemical Analyses

Merit Parameters	Immuno-electrophoresis	Immuno-diffusion
1. Sensitivity	5 - 10 μg protein nitrogen/ml	5 - 10 μg protein nitrogen/ml
2. Sample size	0.004 ml	0.003 ml
3. Time required	20 - 24 hrs.	20 - 24 hrs.
4. Reproducibility	9	8
5. Suitability for null gravity use	4	4
6. Overall safety	4	4
7. Nontoxic, noncaustic reagents	2	2
8. Specificity	10	10
9. Insensitive to environmental changes	6	6
10. Ease in training personnel	8	8
11. Degree of separation required (10=none)	10	10
12. Minimal handling by analyst	7	7
13. Common use of analytic equipment	3	3
14. Nondestructive of sample	0	0
15. Figure of merit	27	24

    2. Discussion

        The coating of plates, or photographic film with agar gel for either of these two procedures can be done easily. The buffered agar gel can be prepared in advance and stored at refrigerator

identification or quantitation of the precipitin bands. When cellulose acetate is used as a medium the washing time after diffusion can be reduced greatly. In order to eliminate staining, and thereby eliminate the use of glacial acetic acid and methanol, recording of results is done by photographing the wet strips, using dark field illumination techniques.[8]

Immunodiffusion appears to be the most practical method for the study of serum complement in this project. Laboratory procedures designed for the measurement of the complement system are intricate.[7] Measurements of complement with immunodiffusion are restricted to the third component of the hemolytic complement system (β_{1C}) in serum. However, in most clinical work there has been good correlation with the use of β_{1C} determinations as an indicator of in vivo antigen-antibody reactions and of hemolytic processes.

Antiserums for these procedures are available commercially. The use of goat antiserum is advised because of the nature of the precipitating antibodies formed in this species of animal.

C. Areas for research and development

The major disadvantage of these procedures in their present status of development is the prolonged diffusion period (20 - 40 hours) required. If these procedures are desired for this project, it would be advantageous to see if any factors in the space flight environment can lead to shortening of the diffusion time.

D. References

1. Cawley, L.P.; Eberhardt, L.; Wiley, J.L.; Double Immunodiffusion with Agar-Coated Plastic Film Base, Vox, Sang., Vol. 10, 1965, pp. 116 - 125.

   The use of agar coated plastic film base for microimmunodiffusion is discussed in this article. The authors point out that a large number of micromethods may be carried out upon a single sheet of plastic.

temperature in covered vials. Usually sodium azide can be added
to the buffer used so as to prevent bacterial growth during storage.
The stored solidified agar can then be remelted in a hot waterbath
(100° C) and then poured on the material that will support the gel.

The original method of immunoelectrophoresis described by
Grabar[5, 6] utilized agar coated photographic glass plates. Micro-
methods are described in which the hot agar gel is molded on 76 x
26 mm glass slides.[10] One major drawback with this modification
is that the area in which the precipitin bands are formed is limited,
thereby decreasing the sensitivity of the procedure. It should be
stated that immunoelectrophoresis approaches the sensitivity of
starch gel electrophoresis while at the same time it is technically
less complicated.

**Personal experience, as well as reports by Cawley et al.[2] ,
have shown that these strips can be manipulated easily because of
their flexibility. In addition, antigen wells for the application of
the serum sample and the antibody trough used for the deposition of**
antiserum are easily made on the agar. Furthermore these film
strips can be treated with several histochemical reagents without
ill effects. These film strips can be kept as permanent records.

Cellulose acetate membranes can be adopted, if desired, as a
medium for the immunoelectrophoretic identification of serum pro-
teins.[4, 9] The patterns on cellulose acetate are generally similar
to those observed on agar gel; however, some differences in position
along the electrophoretic axis are reported. All methods using
cellulose acetate for immunoelectrophoresis require long diffusion
periods (46 hrs. to 72 hrs.)

Immunodiffusion techniques performed in glass colums involve
less manipulation than agar gel strips. This is because the gel
columns do not require washing. Washing of the strips is a neces-
sity for immunoelectrophoresis and immunodiffusion prior to the

2. Cawley, L.P.; Schneider, D.; Simplified Gel Electrophoresis, II Application of Immunoelectrophoresis. J. Clin. Lab. Med., Vol. 65, 1965, pp. 342 - 354.

   This article discusses a simplified system of agar gel electrophoresis in which a low concentration of agar is supported on 35 mm Cronar polyester photographic film base leader strips. While the electrophoresis time is brief (30 to 45 mins.) the diffusion phase takes 24 hours at room temperature.

3. Claman, H.M.; Merrill, D.; Quantitative Measurement of Human Gamma - 2, Beta - 2A, and Beta - 2M Serum Immunoglobulins. J. Lab. and Clin. Med., Vol. 64, 1964, pp. 685 - 693.

   This article presents a modification of Oudin's method of single precipitation in agar for measuring immunoglobulins in serum. The diffusion is carried out in glass tubing filled with agar and antiserum. The method presented is sufficiently simple and reproducible.

4. Feinberg, M.P.; Mann, L.T.; Blatt, W.F.; Cellulose Acetate Media in Immunochemical Tech. Amer. J. Clin. Path., Vol. 44, 1965, pp. 177 - 181.

   The use of cellulose acetate membranes as a medium for immunoelectrophoresis is presented. Details of the apparatus used for electrophoresis and of the preparation of troughs and wells are given. Diffusion time is given 60 to 72 hours at 20° C.

5. Grabar, P.; The Use of Immunochemical Methods in Studies on Proteins. Adv. in Protein Chem., Vol. 13, 1958, pp. 1 - 33.

   This article deals principally with the possibilities of immunochemical methods for the study of serum proteins. The chief limitations of immunochemical methods are given. The chief limitation given is the heterogeneity of the antibodies used.

6. Grabar, P.; Burtin, P.; Immuno-Electrophoretic Analysis, Elsevier Publishing Co., New York, 1965.

   This is a complete monograph dealing with the principles of immuno-electrophoretic analysis and its applications to the study of human plasma in normal and pathologic states.

7. Kabat, E.A.; Mayer, M.M.; Experimental Immunochemistry. Second edition, Charles C. Thomas, Springfield, 1961, chapter 4.

This chapter discusses Complement and the various assay methods. This is a very theoretical presentation.

8. Nace, G. W.; Alley, J. W.; On the Photography of Unstained, Differentially Stained and Fully Stained Precipitation Lines in Agar. J. Biol. Photo. Assoc., Vol. 29, 1961, pp. 125 - 233.

    This paper discusses the use of both transmitted light and dark field illumination for the photography of agar diffusion patterns. The value of the dark field technique is presented.

9. Nelson, T. L.; Stroup, G.; Weddell, R.; Immunoelectrophoretic Identification of Human Serum Proteins on a Cellulose-Acetate Medium. Amer. J. Clin. Path., Vol. 42, 1964, pp. 237 - 244.

    The authors discuss the use of cellulose acetate as a medium for immunoelectrophoresis, emphasizing the small volume required (0.001 ml to 0.005 ml).

10. Wunderly, C.; Immunoelectrophoresis. Adv. in Protein Chem., Vol. 4, 1961, pp. 208 - 273.

    This article is a review of the principles taking part in electrophoretic separation in Agar Gel. The sensitivity of these immunological reactions is given. Macromethods and micromethods of these immunochemical procedures are discussed.

III. The titration of serum anti-A and anti-B isoagglutinins

If a person has blood group A, anti-B agglutinins are present in the serum. A person of blood group B has anti-A agglutinins in the serum. A person of blood group O has both of these agglutinins. Individuals of blood AB have none of these agglutinins. Two molecular types of these agglutinins can be found normally in one individual, namely, the 19S and 7S types.

Complete absence of the agglutinins in persons who should possess them is very rare. This has been observed in hypogammaglobulinemia and in some hematologic diseases. Isoagglutinins may become weak or absent following any injury to the reticuloendothelial system.

A. Principles of present methodology

Present laboratory methods involve the addition of two-fold serial dilutions of serum to an equal volume of a 2 to 5% saline suspension of washed human A or B erythrocytes depending on the blood type of the individual. After the 19S agglutinins are estimated, the serum is neutralized by the addition of purified blood group A or B substances so that the 7S isoantibodies can be titered with antiglobulin serum.

The 19S agglutinins are neutralized by these type specific substances.[3]

B. Suitability of present methodology to space flight conditions

1. Merit table

Anti-A, Anti-B Isoagglutinins

Merit Parameters	Serologic Quantitation of Anti-A and Anti-B Agglutinins
1. Accuracy	approx. 85%
2. Sample size	0.1 ml
3. Time required	1 hour
4. Reproducibility	9
5. Suitability for null gravity use	8
6. Overall safety	10
7. Nontoxic, noncaustic reagents	10

Anti-A, Anti-B Isoagglutinins - continued

Merit Parameters	Serologic Quantitation of Anti-A and Anti-B Agglutinins
8. Specificity	10
9. Insensitive to environmental change	10
10. Ease in training personnel	10
11. Degree of separation required (10=none)	10
12. Minimal handling by analyst	6
13. Common use of analytic equipment	0
14. Nondestructive of sample	0
15. Figure of merit	70

2. Discussion

This method while relatively simple requires a considerable degree of manipulation. This is because of the serial dilutions of the serum sample which have to be made. At best, this type of serologic test renders only an estimate of the 19S and 7S agglutinins present. The quantitative precipitin method[1] for determination of these agglutinins while more accurate than the method presented here is not practical for this project. This is because of the time requirements and of the manipulation involved.

The commercial preparations of antiserums (anti-A, anti-B, antiglobulin) and of type specific substances A and B can be used for this determination.

C. Areas for research and development

These areas do not necessarily involve this quantitation. The use of latex particles coated[2] with appropriate antigens can be investigated for the detection of other antibodies in the serum. These procedures are based either on the principle of agglutination - inhibition or direct agglutination. These tests could be employed as simple screening procedures or they can be designed for the serologic quantitation of a given substance in the serum. Applications of these tests

have been made in mycology, parasitology, and in general immu-
nology.

D. References

1. Kabat, E.; Bezer, A. E.; Immunochemical Studies on Blood Groups:
   I. Estimation of A and B Isoantibodies in Human Serum by the Quan-
   titative Precipitin Method. J. Exper. Med., Vol. 82, 1945, pp. 20
   pp. 207 - 215.

   The authors discuss a microprecipitin method for the quantita-
   tation of A or B isoantibodies in serum. The results are given in
   amounts of nitrogen precipitated with each type specific substance.
   The precipitation phase as presented in the paper involves a long
   period of time.

2. Kabat, E. A.; Mayer, M. M.; Experimental Immunochemistry.
   Second Edition. Charles C. Thomas, Springfield., 1961, p. 124.

   The use of polystyrene latex particles in agglutination procedures
   is presented. Diagnostic areas in which these particles have been
   used are given.

3. Mollison, P. L.; Blood Transfusion in Clinical Medicine, F. A.
   Davis, Co., Philadelphia, 1967, pp. 246 - 255.

   In the section noted here the author gives a thorough explana-
   tion of the ABO Blood System. Methodology is discussed briefly.

3.3.07 Methemoglobin

The measurement of methemoglobin in blood is rather difficult especially when this derivative of hemoglobin is present in small amounts. The only chemical difference between hemoglobin and methemoglobin is that in the former the iron atom is in the ferrous state (Fe^{++}) while in the latter the iron is oxidized to the ferric ion (Fe^{+++}).

A. Principles of present methodology

Method 1: Evelyn and Malloy[1]

Principle: Methemoglobin has an absorption maximum at 630 mμ. Addition of NaCN converts methemoglobin to cyanmetemoglobin and the absorption peak at 630 mμ disappears almost completely. The difference in absorbance at 630 mμ (A_{630}) is directly proportional to the concentration of methemoglobin.

Procedure: A sample of blood is diluted 1:51 with phosphate buffer, pH 6.6 and is allowed to lake. The solution is centrifuged to remove the cell debris and the absorbance A_1 of the supernatant is measured at 630 mμ against water. A drop of cyanide is added to the solution and its absorbance A_2 is measured again at the same wavelength. The difference of the two absorbances ($A_1 - A_2$) is proportional to the concentration of methemoglobin.

Turbidity due to lipemic blood samples will not affect the results.

Method 2: Martinek[4]

Principle: The determination of methemoglobin is made by measuring the absorbance of a diluted blood sample at two wavelengths. The two wavelengths λ_1 and λ_2 are selected in such a way that at λ_1 the ratio of the absorbances of methemoglobin to oxyhemoglobin, is the highest and at λ_2 the ratio is 1.0 (isobestic point). The calculation of methemoglobin concentration involves the solution of simultaneous equations.

Procedure: Heparinized blood is diluted 1:101 with distilled water and the the mixture is allowed to stand for a few minutes for complete

hemolysis. The cell fragments are removed by slow speed centrifugation and the absorbance of the clear supernatant is measured at 620 and 530 mμ. The amount of methemoglobin is expressed as a percentage of the total hemoglobin in blood.

Lipemia does not interfere with this method. It is preferable to use a buffer to control the pH; distilled water with a pH below 5 and above 7 is unsatisfactory.

The results obtained are in good agreement with those of Method 1.

Similar spectrophotometric techniques have been developed by other investigators. Zijlstra and Muller[5] prefer measurements of the absorbance at 558 and 523 mμ, while Hutchinson[3] suggested the wavelengths of 620 and 520 mμ.

B. Suitability of present methodology to space flight conditions

    1. Merit table

Methemoglobin

Merit Parameters	Method 1	Method 2
1. Sensitivity	.2 g	.2 g
2. Sample size	.1 ml	.05 ml
3. Time required	20 min	15 min
4. Reproducibility	7	7
5. Suitability for null gravity use	9	9
6. Overall safety	2	10
7. Nontoxic reagents	2	10
8. Noncaustic reagents	10	10
9. Specificity	9	8
10. Insensitive to environmental changes	2	9
11. Ease in training personnel	9	9
12. Degree of separation required (10=none)	8	8
13. Minimal handling by analyst	7	8
14. Common use of analytic equipment	10	10

Methemoglobin - continued

Merit Parameters	Method 1	Method 2
15. Nondestructive of sample	0	2
16. Figure of merit	26	59

2. Discussion

The accuracy of both methods is not very good when the methemoglobin level is within the normal range, up to 0.5 g/100 ml of blood.

The method of Evelyn and Malloy is preferred by many investigators but it suffers from certain disadvantages, i.e., the high intoxicity of NaCN reagent. Perhaps the NaCN can be replaced by sodium azide which is far less toxic and quite stable.

The differential spectrophotometric method is more attractive and easier to perform. It should be emphasized that the validity of this method depends on the presence of no more pigments in blood than are assumed to be present.

Further experimentation will be required to determine the optimum wavelengths for the spectrophotometric procedures. The method of Martinek[4] or some other modification[3,5] is preferable for the present time.

Equipment

Method 1: Spectrophotometer, centrifuge

Method 2: Spectrophotometer, centrifuge

C. Areas for research and development

It may also be possible to measure the methemoglobin content of blood by electrophoretic techniques.

D. References

1. Evelyn, K.A.; Malloy, H.T.; Micro-determination of Oxyhemoglobin Methemoglobin and Sulfhemoglobin in a Single Sample of Blood. J. Biol. Chem., Vol. 126, 1938, pp. 655 - 662.

Describes the simultaneous determination of oxyhemoglobin and some of its derivatives.

2. Henry, R. J.; Determination of Methemoglobin and Sulfhemoglobin in Clinical Chemistry: Principles and Technics, Harper and Row Publishers, New York, 1964, pp. 75 - 83.

A book devoted entirely to the principles and techniques of chemistry. The author generally discusses several methods in some detail and then selects one or two and describes them in great detail. Provides values of accuracy, precision and normal values for selected procedures.

3. Hutchinson, E. B.; The Measurement of Methemoglobin, Am. J. Med. Tech., Vol. 26, 1960, pp. 75 - 83.

Methemoglobin is measured in blood by direct spectrophotometry in two wavelengths.

4. **Martinek, R. G.; Spectrophotometric Determination of Abnormal Hemoglobin Pigments in Blood. Clin. Chim., Acta 11, 1965, pp. 146 - 158.**

Methemoglobin is measured spectrophotometrically in a diluted blood sample without any additions.

5. Zijlstra, W. G.; Muller, C. J.; Spectrophotometry of Solutions Containing Three Components with Special Reference to the Simultaneous Determination of Carboxyhemoglobin and Methemoglobin in Human Blood. Clin. Chim. Acta, Vol. 2, 1957, pp. 237 - 245.

Method for methemoglobin by spectrophotometry.

3.3.08 Mucoproteins

Many complexes of carbohydrate with proteins are classified as mucoproteins and glycoproteins.

Mucoproteins are characterized by the fact that the carbohydrate-protein linkage is easily broken by hydrolysis or by an electric field suggesting an ionic or polar-type linkage.

Glycoproteins are defined as having at least 0.5% hexosamine and having no hexuronic or sulfate esters. The carbohydrate-protein bond can be split by drastic treatment, strong alkali, or acid.

Mucoids are defined as mucoporteins having at least 70% carbohydrate while mucoproteins have considerably less.

The fraction which is usually isolated from serum by precipitation with phosphotungstic acid, after removal of the serum proteins by perchloric acid, is called seromucoid.

Two fractions are usually measured in serum, (1) Seromucoid, (2) Protein- bound Hexose.

A. Principles of present methodology

  1. Seromucoid in serum[2]

     Principle: Serum proteins are precipitated with 1.2 $\underline{M}$ perchloric acid. After removal of the precipitate by centrifugation, the seromucoid is precipitated in the supernatant by phosphotungstic acid. The precipitated seromucoid is dissolved in dilute NaOH and quantitated colorimetrically at 485 or 520 mμ by the orcinol reaction. Results are expressed as mg of seromucoid per 100 ml of serum in terms of a galactose-mannose standard.

  2. Protein-bound hexose in serum[3]

     Principle: Protein-carbohydrate conjugates containing hexose are precipitated from serum by addition of ethanol. The hexose is measured in the precipitate by the orcinol reaction.

  3. Acid mucopolysaccharides (AMP) in urine[1]

     Principle: Uromucoid and acid mucopolysaccharides are

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selectively precipitated from urine by cetyltrimethyl-ammonium bromide (CTV). The precipitates are centrifuged, the supernatant is discarded and the CTV-AMP complexes are dissociated by treatment with ethanol saturated with NaCl. The AMP is dissolved in NaOH and the various carbohydrate moieties are measured by color reactions employing specific reagents.

B. Suitability of present methodology to space flight conditions

1. Merit table

Mucoproteins

Merit Parameters	Sero-Mucoid	Protein-Bound Hex.	AMP Urine
1. Sensitivity	-	-	-
2. Sample size	0.5 ml	0.1 ml	40 ml
3. Time required	1 hour	1 hour	2 days
4. Reproducibility	6	6	6
5. Suitability for null gravity use	-	-	-
6. Overall safety	0	0	0
7. Nontoxic reagents	5	5	5
8. Noncaustic reagents	0	0	0
9. Specificity	7	7	7
10. Insensitive to environmental changes	6	6	6
11. Ease in training personnel	2	4	1
12. Degree of separation required (10=none)	4	6	3
13. Minimal handling by analyst	2	3	1
14. Common use of analytic equipment	9	9	9
15. Nondestructive of sample	1	1	2
16. Merit range	11	6-13	5-11
17. Mean figure of merit	8	9	8

2. Discussion

Strong sulfuric acid is required for the color reactions involved in the methodology presented above with the exception of Protein-

Bound Hexose which can be measured by turbidimetry.

Equipment

All three determinations: heating bath, centrifuge, spectrophotometer

C. Areas for research and development

Electrophoretic and immunological studies may prove to be valuable in these determinations. Infrared spectra of isolated fractions may also provide valuable information.

D. Comments

No method of any kind can be recommended until it is defined what type of polysaccharide is to be measured.

E. Reference

1. Clausen, J.; Asboe-Hansen, G.; Urinary Acid Mucopolysaccharides in Mastocytosis. A New Technique for Quantitative Estimation. Clin. Chim. Acta, Vol. 13, 1966, pp. 475 - 483.

   Acid mucopolysaccharides are precipitated by cetyltrimethyl-ammonium bromide and quantitated colorimetrically.

3.3.09 <u>Serum proteins</u>

   I.   Serum albumin

       A. Principles of present methodology

          1.   Method 1

Albumin can be estimated by any one of the numerous electrophoretic techniques with simultaneous determination of the **total** serum proteins or by one of the three methods described under part II, "Total Serum Proteins." Ultraviolet scanning of the electropherograms permits the estimation of albumin as percentage of the total serum protein without drying or staining.[2]

          2.   Method 2

Albumin can also be measured by a dye binding method. Rodkey[3] proposed the use of bromcresol green for the estimation of serum albumin. Unlike other dyes, bromcresol green binds specifically with albumin and there is no interference from other serum proteins. Albumin values by this dye-binding method are in very good agreement with those obtained with electrophoretic techniques. Bartholomew[1] modified Rodkey's method and developed an excellent procedure for the measurement of serum albumin. When serum is added to a solution of bromcresol green in 0.05 <u>M</u> citrate buffer, pH 4.0, the optical density of the solution increases and this increase is directly proportional to the albumin concentration. Absorbance measurements are made at 620 - 640 mμ. The method is very sensitive, specific and can be easily automated.

       B. Suitability of present methodology to space flight conditions

          1.   Merit table

<div align="center">Serum Albumin</div>

Method Parameters	Method 1	Method 2
1. Sensitivity	Good	Excellent
2. Sample size	1 μl	10 μl
3. Time required	45 min	20 min
4. Reproducibility	7	9

<div align="center">78</div>

Serum Albumin - continued

Method Parameters	Method 1	Method 2
5. Suitability for null gravity use	-	9
6. Overall safety	6	10
7. Nontoxic reagents	-	10
8. Noncaustic reagents	9	10
9. Specificity	10	10
10. Insensitive to environmental changes	-	6
11. Ease in training personnel	5	9
12. Degree of separation required (10=none)	9	9
13. Minimal handling by analyst	2	8
14. Common use of analytic equipment	5	10
15. Nondestructive of sample	0	0
16. Merit range	14-45	-
17. Mean figure of merit	27	75

2. Discussion

Both methods are very suitable for the measurement of serum albumin; however, the dye-binding method is preferable because of its simplicity, specificity, and excellent reproducibility. The compiler believes this is the method of choice.

Equipment

Method 1: Electrophoresis apparatus

Method 2: Spectrophotometer, centrifuge, automatic diluting pipette.

C. Areas for research and development

(None)

D. References

1. Bartholomew, R. J.; Sulphonephthaleins are Specific Reagents for Albumin. VI International Congress of Clinical Chemistry (Abstract) Enzym. Biol. Clin., Vol. 6, 1966, p. 168.

   Bromcresol green at an acid pH is a specific reagent for albumin.

2. Ressler, N.; Photoelectric Ultraviolet Photometry Applied to Protein Electrophoresis. J. Lab. Clin. Med., Vol. 54, 1959, pp. 291-299.

   Electrophoretic patterns are scanned at 200 mμ without drying or staining. The technique permits the estimation of albumin and other serum protein fractions.

3. Rodkey, F. L.; Direct Spectrophotometric Determination of Albumin in Human Serum. Clin. Chem., Vol. 11, 1965, pp. 478 - 487.

   Serum albumin is measured directly by a dye-binding method with bromcresol green.

II. Total serum proteins

There are several methods which permit rapid and reliable measurements of the total serum proteins.

A. Principles of present methodology

1. Refractometry

The estimation of serum proteins by measurement of the refractive index was investigated by Sunderman[5] and has been reviewed by Naumann.[4]

Most of the commercially available instruments require a sample of serum from 0.05 to 0.30 ml. The protein content of serum is given by the following equation.[5]

Protein content in $g\% = 510 \, (RI_S - RI_{H_2O}) - 1.81$

where: RI_S = the refractive index of serum

RI_{H_2O} = the refractive index of pure water.

The above equation has recently been corrected by Sundermann[6] as follows:

Protein content in $g\% = 533.6 \, (RI_S - RI_{H_2O}) - 1.89$

The correction is valid and based on the fact that the Kjeldahl nitrogen factor for serum proteins is 6.54 rather than the traditionally accepted value of 6.25.

The results obtained by refractometry are in good agreement with those of the biuret and Kjeldahl techniques.[2,4] The same serum sample may be used for analysis of other serum components. A possible source of error is the temperature effect but that is negligible in methods determining the difference of the refractive indices of serum and water as long as both are at the same temperature.[2,5]

The method has good precision, adequate sensitivity, and does not require highly trained personnel.

280 mμ due to the presence of aromatic amino acids. The intensity of absorption in this spectral region depends on the aromatic amino acid content of a particular protein and various proteins differ widely in their specific absorptivity. However, in the spectral region between 200 and 240 mμ, absorption of light is principally due to peptide bonds and proteins differ relatively little in their specific absorptivity. Furthermore, the absolute absorbance in the 200 - 240 region is much greater than at 260 - 280 mμ.

In a procedure proposed by Tombs, et al.[7], serum is diluted 1:2000 with 0.9 percent sodium chloride solution and absorbance measurements are made at 210 mμ. A linear relationship exists between protein concentration and optical density.

The specific extinction of total serum protein at 210 mμ is:

$$E^{1\%}_{1\ cm} = 205 \pm 1.4$$

In the method developed by Waddell[8] total serum proteins are determined from the difference in absorbances of a 1:1000 dilution of serum at 215 and 225 mμ. The use of the difference, rather than of the absorbance at a single wavelength, almost eliminates the error from non-protein serum constituents, since the difference in absorbance, at these two wavelengths, of human plasma ultrafiltrates was found to be negligible. Moreover, the use of somewhat longer wavelengths in the Waddell method results in significantly lower stray light errors than those that exist at 210 mμ.

Both methods are highly sensitive and require very small serum samples. The high dilution of serum eliminates essentially all potential interference from non-protein substances absorbing between 215 and 225 mμ.

To minimize potential errors, the serum dilution must be prepared with extreme care and the volume of the sample must be measured with meticulous attention. Highly accurate pipettes

should be employed; a well-calibrated mechanical pipetting-diluting machine would be preferable to hand pipetting.

3. Biuret

This method is probably the most widely used for the determination of total serum proteins, because of its simplicity and reproducibility.

Principle: A sample of serum is mixed with the biuret reagent[3] and the optical density of the resulting colored complex is measured at 545 mμ. The optical density is directly proportional to the protein content of serum. Many biuret reagents have been proposed but the one described by Huerga et al.[1] has been found to be very stable. It incorporates copper sulfate, sodium-potassium tartrate, sodium hydroxide and potassium iodide in a single solution.

B. Suitability of present methodology to space flight conditions

1. Merit table

Total Serum Proteins

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	.05 g	1 μg	.05 g
2. Sample size	.05 ml	1-5 μl	20 μl
3. Time required	2 min	5 min	20 min
4. Reproducibility	9	9	9
5. Suitability for null gravity use	10	9	9
6. Overall safety	10	10	6
7. Nontoxic reagents	10	10	7
8. Noncaustic reagents	10	10	5
9. Specificity	8	8	10
10. Insensitive to environmental changes	9	9	6
11. Ease in training personnel	10	8	9
12. Degree of separation required (10=none)	9	9	9
13. Minimal handling by analyst	9	6	9
14. Common use of analytic equipment	2	9	10

Total Serum Proteins - continued

Merit Parameters	Method 1	Method 2	Method 3
15. Nondestructive of sample	10	0	0
16. Figure of merit	82	73	50

2. Discussion

Any of the three described methods may be applicable to space flight conditions. Method 1 is preferred if an instrument capable of measuring the refractive index with a precision of 0.0001 unit is available. The required handling of the specimen is minimal and no reagents are necessary.

Lipemic sera must be avoided with any of the three methods. The length of time for analysis listed under each method does not include the time required for centrifugation of the specimen to accomplish the separation of serum from the blood clot.

Equipment

Method 1: Refractometer, centrifuge

Method 2: Ultraviolet spectrophotometer, centrifuge automatic pipetting-diluting equipment

Method 3: Spectrophotometer of photoelectric colorimeter, centrifuge

It is possible to avoid the high dilution in the spectrophotometric method by using cuvettes of a very short light path.

C. Areas for research and development

Absorption spectra of sera in the ultraviolet may provide additional useful information. Specific immunologic reactions may also be employed to quantitate specific proteins either by precipitation techniques or by more sensitive means such as fluorescence depolarization.

D. References

1. De la Huerga, J.; et al., In Serum Proteins and the Dysproteinemias, J.B. Lippincott, Co., Philadelphia, 1964, pp. 52 - 62.

Discusses the determination of serum proteins by the biuret method.

2. Drickman, A.; McKeon, F.A.; Determination of Total Serum Proteins by Means of the Refractive Index of Serum, Am. J. Clin. Path., Vol. 38, 1962, pp. 392 - 396.

   Compares values of serum protein obtained with the biuret method and refractometry.

3. Kingsley, G.R.; The Direct Biuret Method for the Determination of Serum Proteins as Applied to Photoelectric and Visual Colorimetry, J. Lab. Clin. Med., Vol. 27, 1942, pp. 840 - 845.

   Application of the biuret reaction for the determination of serum proteins.

4. Naumann, H.N.; In Serum Proteins and the Dysproteinemias, J.B. Lippincott Co., Philadelphia, 1964, pp. 86 - 101.

   Principles of determination of serum proteins by refractometry.

5. **Sunderman, F.W.; A Rapid Method for Estimating Serum Proteins. Formula for Calculating Serum Protein Concentration from the Refractive Index of Serum, J. Biol. Chem., Vol. 153, 1944, pp. 139 - 142.**

   The Abbé refractometer was used for the determination of serum **proteins.**

6. Sunderman, F.W.; Revised Calculations for Total Serum Proteins by Refractometry and for Total Base (and Sodium) by Conductivity, Am. J. Clin. Path., Vol. 46, 1966, pp. 679 - 683.

   The use of the Kjeldahl N factor of 6.54 requires revision of refractometry formulas.

7. Tombs, M.P., et al., The Spectrophotometric Determination of Protein at 210 mμ, Biochem. J., Vol. 73, 1959, pp. 167 - 171.

   Absorption measurements of diluted serum at 210 mμ can be employed for the estimation of total proteins.

8. Waddell, W.J.; A Simple Ultraviolet Spectrophotometric Method for the Determination of Protein, J. Lab. Clin. Med., Vol. 48, 1956, pp. 311 - 314.

   Describes a micromethod for the determination of serum protein by ultraviolet spectrophotometry.

3.3.10 <u>Transferrin</u>

Transferrin is a serum protein which combines with iron and serves as a vehicle for iron transport. Approximately 25 - 40% of the transferrin exists in serum in combination with iron and the remainder is free transferrin. Concentrations of transferrin are expressed in terms of micrograms of iron per 100 ml of serum under conditions of full saturation.

The maximum amount of iron that can be bound to transferrin is known as Total Iron Binding Capacity (TIBC). The amount of free transferrin in serum is known as Unsaturated Iron Binding Capacity (UIBC).

There are three approaches for estimating the concentration of transferrin in serum. Two of them are chemical and the third immunochemical.

A. Principles of present methodology

    1. Williams and Conrad[4]

        a. Principle

This approach measures serum iron (SI) and the amount of free transferrin (UIBC). Then SI + UIBC = TIBC. The UIBC can be measured by adding a known quantity of iron to saturate the transferrin and then measure the excess iron with a reagent which reacts with the free iron but not with the iron bound to transferrin.

        b. Method

The pH of serum is lowered to approximately 2.2 by addition of a KCl-HCl buffer containing hydroxylamine hydrochloride as the reducing agent. At this low pH the iron is released from the transferrin and upon addition of 2,4,6-tripyridyl-s-triazine (TPTZ), it reacts to give a colored complex the absorbance of which is measured at 595 mμ. The absorbance reading is used to determine the iron concentration of the serum. The same solution is used for the determination of UIBC.

The solution is made alkaline, (pH 8-9) by the addition of "Tris" buffer. At this pH the iron dissociates from TPTZ and recombines with the transferrin. A known amount of iron is now added to the solution to saturate the transferrin. The excess iron is measured by the addition of 2, 2', 2" tripyridyl which reacts only with the free iron to give a colored complex the absorbance of which is measured at 552 mμ. The UIBC is determined by subtracting the excess iron from the known amount which was added to the solution. Both determinations of SI and UIBC are performed in a single tube. Addition of SI and UIBC gives the TIBC. Reliable serum iron concentrations and UIBC can be obtained in specimens containing up to 15 mg% of bilirubin or 150 mg% of hemoglobin.

2. Goodwin, et al.[2]

   a. Principle

      Iron in excess is added to serum and allowed to combine with transferrin. The unbound iron is removed by addition of solid magnesium carbonate. After removal of the magnesium carbonate by centrifugation, the amount of iron is measured in the supernatant. The content of iron in micrograms per 100 ml of serum represents the TIBC.

   b. Method

      A known quantity of iron is added in excess to a serum sample to saturate the transferrin. The unbound iron is precipitated by the addition of solid $MgCO_3$. The mixture is centrifuged to remove the $MgCO_3$. The iron content of the supernatant solution is measured and this represents the total iron binding capacity (TIBC). The supernatant solution is mixed with acetate buffer, pH 4.5, to release the iron from the transferrin. The liberated iron is complexed with 4, 7-diphenyl-1, 10-phenathroline disulfonic acid, disodium salt.

The resulting pink color is measured at 535 mμ. The procedure requires 1 ml of serum but it can be scaled down so that a volume of 40 μl is adequate.[3]

3. Burrows[1]

a. Principle

Serum is placed in immuno-diffusion plates containing antiserum specific for human transferrin. A precipitating ring is formed in the agar gel.

b. Method

This procedure employs the "Immuno-Plate"* which is an agar gel diffusion plate containing antiserum specific for human transferrin. Serum is placed in small wells of the diffusion plate and incubated at 37° C for 4 hours. The diameter of the precipitin ring around each well is measured. The concentration of transferrin in the serum is determined by comparison with standard solutions of transferrin treated in the same manner. The concentration of transferrin is proportional to the diameter of the precipitin ring formed, and it is expressed in micrograms of iron-binding capacity per 100 ml of serum.

This method is simple and very specific but unfortunately grossly underestimates the iron-binding capacity of serum as judged by comparison with results by other methods. However, the approach is sound and it is possible that the method can be improved.

B. Suitability of present methodology to space flight conditions

1. Merit table

Transferrin

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	-
2. Sample size	.05 ml	1 ml	-

* "Immuno-Plate" is a registered trademark of Hyland Laboratories, Los Angeles, California.

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Transferrin - continued			
Merit Parameters	Method 1	Method 2	Method 3
3. Time required	1 hour	1 hour	4 hours
4. Reproducibility	8	8	-
5. Suitability for null gravity use	8	8	8
6. Overall safety	10	10	10
7. Nontoxic reagents	9	9	10
8. Noncaustic reagents	9	9	10
9. Specificity	8	8	9
10. Insensitive to environmental changes	8	7	-
11. Ease in training personnel	4	3	8
12. Degree of separation required (10=none)	9	9	9
13. Minimal handling by analyst	5	4	8
14. Common use of analytic equipment	10	10	10
15. Nondestructive of sample	1	1	1
16. Merit range	-	-	0-82
17. Mean figure of merit	55	54	38

2. Discussion

Method 1 is preferable because all determinations are performed in a single tube. All optical measurements can be made in the reaction tube or cuvette. Fasting blood samples should be obtained because lipemic sera may seriously interfere. Hydroxylamine hydrochloride may be used as a reducing agent because it is far more stable than ascorbic acid.

Equipment

Method 1 and 2: Spectrophotometer, disposable iron-free glassware or plastic tubes, centrifuge, incubator.

Method 3:   a. "Immuno-Plates"

         b. Incubator

C. Areas for research and development

Method 3 should be investigated; its simplicity can provide a very

suitable procedure.

D. References

1. Burrows, S.; Comparison of Methods Designed to Measure Transferrin and Iron-Binding Capacity of Serum. Am. J. Clin. Path., Vol. 47, 1967, pp. 326 - 328.

   Compares values of transferrin by an immunological method and by procedures measuring total iron-binding capacity.

2. Goodwin, J.F.; Murphy, B.; Guillemette, M.; Direct Measurement of Serum Iron and Binding Capacity, Clin. Chem., Vol. 12, 1966, pp. 47 - 57.

   Serum iron, total iron binding capacity, and unsaturated iron binding capacity are measured with bathophenanthroline.

3. Watkins, D.K.; Butler, E.B.; Micromethod for the Determination of Serum Iron and Total Iron-binding Capacity and its Application in Pregnancy, Clin. Chim. Acta, Vol. 13, 1966, pp. 448 - 456.

   TIBC is measured in 40 μl of serum.

4. Williams, H.L.; Conrad, M.E.; A One Tube Method for Measuring the Serum Iron Concentration and Unsaturated Iron-Binding Capacity, J. Lab. Clin. Med., Vol. 67, 1966, pp. 171 - 176.

   Serum iron and UIBC are measured without precipitating the serum proteins.

3.3.11 Urinary proteins

In normal individuals the amount of protein excreted in the urine is undetectable by routine methods. A qualitative test for urinary protein is probably more than adequate.

A. Principles of present methodology

1. Kutter[2]

The simplest way to detect protein in the urine is probably by means of dipsticks supplied by Ames Company, under the trade-names "Hema-Combistix" and "Labstix." These dipsticks are quite sensitive in that they can detect protein levels as low as 7 mg/100 ml urine.[2] They suffer from the disadvantage of reacting only with albumin, but proteinuria without albuminuria will be encountered very rarely.

Both dipsticks are paper strips impregnated with bromphenol blue buffered at an acid pH. In the presence of albumin bromphenol blue undergoes a color change from yellow to blue. Semi-quantitative results can be obtained with the dipsticks. A false positive result may be obtained with stale urines or urine specimens with high alkalinity.

2. Dannenberg[1]

Another convenient way for the detection of urinary proteins had been proposed by Dannenberg.[1] A small volume of urine is added to a tube containing a dry mixture consisting of 85 parts sea sand and 15 parts of crystalline sulfosalicyclic acid. The supernatant will show turbidity if the urine contains protein. However, under null gravity conditions low speed centrifugation will be required to separate the sand from the turbid supernatant.

B. Suitability of present methodology to space flight conditions

1. Merit table

Urinary Proteins

Merit Parameters	Kutter	Dannenberg
1. Sensitivity	7 mg%	5 mg%
2. Sample size	0.1 ml	0.5 ml
3. Time required	0.5 min	10 min
4. Reproducibility	-	-
5. Suitability for null gravity use	10	3
6. Overall safety	10	5
7. Nontoxic reagents	10	9
8. Noncaustic reagents	10	5
9. Specificity	9	9
10. Insensitive to environmental changes	8	9
11. Ease in training personnel	10	10
12. Degree of separation required (10=none)	10	10
13. Minimal handling by analyst	10	3
14. Common use of analytic equipment	10	1
15. Nondestructive of sample	10	0
16. Merit range	0-96	0-35
17. Mean figure of merit	48	18

2. Discussion

It seems that the use of dipsticks is the method of choice. Hundreds of paper strips can be stored in a small bottle for long periods of time.

Another advantage of the dipstick is that one can also test for ketone bodies (acetoacetic acid and acetone), glucose, hemoglobins, and pH of the urine.

C. Areas for research and development

(None)

D. References

1. Dannenberg, E.; Rapid Analysis: Detection of Protein with Dry Reagent, Munch. Med. Wochschr., Vol. 95, 1953, p. 102.

Urine protein can be detected by the addition of urine to a dry mixture of sea sand and sulfosalicylic acid. Turbidity in the supernatant solution indicates protein.

2. Kutter, D.; Simultaneous Determination of Glucose, Protein and pH in Urine. Therap. Gegenwart, Vol. 104, 1965, pp. 496 - 498; 506 - 507.

Evaluates the "Hema-Combistix" for the detection of protein in urine.

3. 3. 12 <u>Adrenocorticotrophic hormone (ACTH)</u>

    A. Principles of present methodology

        There are three estimations which depend on various biological assays. These include the (1) repair test-determination of the re-appearance of lipid material in regressed adrenal cortices of hypo-physectomized rats following injections of preparations containing ACTH; (2) maintenance test-estimation of quantity of ACTH material required to maintain the adrenal weights of rats injected immediately after hypophysectomy; (3) decrease in adrenal ascorbic acid - one hour after i. v. injection of ACTH containing material the decrease in ascorbic acid content of the adrenal glands of a hypophysectomized rat is determined.

        In man various indirect tests have been proposed including: meas-urement of blood and urine levels of corticosteroid following (1) ACTH injection; (2) blockage of ACTH secretion by drugs (SU-4885-Ciba); (3) following administration of a synthetic corticosteroid.

    B. Applicability of present methodology to space flight conditions

        The presently available assays seem to be technically impossible under present space flight conditions.

    C. Areas for research and development

        At the present time there is no practical clinical laboratory pro-cedure[1, 3, 4, 5] available for adrenocorticotrophin on earth -- much less under conditions of space flight. However, two approaches may be studied. The indirect approach requires the development of method-ology for corticosteroid determination under space flight conditions. The second approach would be the development of radioimmunologic assay[2] as described by Rosselin, et al. This method would not apply any new physical principles but depends on the preparation of iodinated I^{131} ACTH and the production of specific antibodies before measuring ACTH in plasma.

D. References

1. DeBarbieri, A.; Urinary Corticotropin, Folia Endocrinol., Pisa, Vol. 7, 1954, pp. 719 - 726.

   Active corticotropic substance was extracted from 200 - 300 liters of urine and assayed by decrease of ascorbic acid in the adrenal gland of hypophysectomized rats.

2. Rosselin, G., et al.; Radioimmunologic Assay of Protein Hormones by the Method of Berson and Yalow. Assay of Protein and of their Antibodies in the Plasma of Man, Vol. 26, 1965, pp. 449 - 464.

   A number of protein hormones in plasma may determine precisely and specifically by radioimmunological techniques. The techniques can be used to measure antibodies to hormones, in particular antibodies to insulin.

3. Rubin, B. L., et al.; Adrenocorticotropic Hormone in Urine, J. Clin. Endocrinol. and Metabolism, Vol. 14, 1954, pp. 154 - 169.

   Fresh urine samples were tested for ACTH activity by i.v. injection into hypophysectomized rats. Minimum amount of ACTH detected after injection was 0.004 I.U.

4. Ruf, K.; Determination of ACTH in Man, Schweiz. Wochschr., Vol. 96, 1966, pp. 684 - 687.

   A review with 68 references.

5. Sayers, G.; Blood Adrenocorticotropin (ACTH), J. Clin. Endocrinol., Vol. 15, 1955, pp. 751 - 759.

   A review of the difficulties encountered in the determination of ACTH in blood. 22 references.

3.3.13 <u>Antidiuretic hormone</u> (ADH)

    A. Principles of present methodology

        Pressor activity is assayed by comparing the rise in blood pressure following i.v. injection in anesthetized dogs or rats with that produced by known amounts of a standard. Antidiuretic activity is assayed by comparing the reduction of urine volume following i.v. injection of an extract in hydrated unanesthetized rats or rabbits with that produced by a standard preparation.

    B. Applicability of present methodology to space flight conditions

        Biological assays seem to be technically impossible under space flight conditions within the next three years. The extremely small quantities of the hormone which may be found in either plasma or urine make it quite unlikely that a method based on physical measurements will be developed in the foreseeable future.

    C. Areas for research and development

        It may be possible that the immunochemical approach will be productive. Sylvana now has available Guinea pig anti-human growth hormone which is sensitive enough to detect 0.025 millimicrograms of human growth hormone using a trace label of I^{131} labeled growth hormone for radioimmunoassay. The reaction between "unknown" serum and the antibody must be carried out for 7 days at 4° C. It seems possible that in time other antibodies will become available. There is no way to estimate development time in this area.

    D. References

        1. Bisset, G.W.; Assay of Oxytoxin and Vasopressin in Blood and the Mechanism or Inactivation of these Hormones by Na Thioglycolate. Oxytoxin, Proc. Intern. Symp., Montevideo, 1959, (Pub. 1961), pp. 380 - 399.

           This is a review article containing some new material on experiments performed on the response of isolated strips of rat uterus.

        2. Ginsberg, M.; A Method for the Assay of Antidiuretic Activity, Brit. J. Pharmacol., Vol. 6, 1951, pp. 411 - 416.

A method based on a four point assay using a regimen of water administration described by Birnie, et al. , (Chemical Abstracts, Vol. 43, p. 3084e.)

3. Hickey, R. C.; Hare, K.; The Renal Excretion of Chloride and Water in Diabetes Insipidus, J. Clin. Invest., Vol. 23, 1944, pp. 768 - 775.

The functional capacity of the neurohypophysis is tested by following the liberation of the antidiuretic hormone in response to the injection of hypertonic saline.

4. Tsukamoto, S.; Antidiuretic Substance in Serum. I. Antidiuretic Substance and its Diurnal Variation. II. Antidiuretic Substance in Urine and its Physiological and Pathological Significance. Kumanoto Daigaku Taishitsu Igaku Kenkyusho Hokoku, Vol. 13, 1962, pp. 236 - 255.

Variation of the "antidiuretic substance" with time of day and season of the year. Diurnal variation was observed.

3.3.14 Aldosterone

A. Principles of present methodology

Current methods depend on solvent extraction of the steroid, followed by chromatographic isolation. Even under ideal laboratory conditions, reproducible results are difficult to obtain. No well developed methods appear to be available which would be directly applicable in a space environment. Gas chromatography appears to be the method that presently offers the most promise.[1,2,3]

B. Suitability of present methodology to space flight conditions

1. Merit table

<div align="center">Aldosterone</div>

Merit Parameters	Gas Chromatography
1. Sensitivity	0.4 μg
2. Sample size	100 ml
3. Time required	> 1 hr
4. Reproducibility	5
5. Suitability for null gravity use	0
6. Overall safety	0
7. Nontoxic reagents	0
8. Specificity	5
9. Insensitive to environmental changes	4
10. Ease in training personnel	1
11. Degree of separation required (10=none)	0
12. Minimal handling by analyst	0
13. Common use of analytic equipment	5
14. Nondestructive of sample	0
15. Figure of merit	3

2. Discussion

Use of albumin (as described in Section 3.3.15 on 17-hydroxy-corticosteroids) might circumvent the need to use organic solvents

in the extraction procedure.

The author of this report is of the opinion that the satisfactory analysis of aldosterone is contingent on the development of new methods.

C. Areas for research and development

1. Isotope dilution method similar to that described for 17-OH-CS in which albumin, which has a strong binding affinity for aldosterone is substituted for CBG.

2. Immunochemical investigation of natural and synthetically prepared aldosterone-protein conjugates.

3. Investigation of the optical rotatory dispersion spectrom of aldosterone and its simple derivatives.

4. Enzymatic methods. Search for enzymes specific for aldosterone.

D. References

1. Kliman, B.; Foster, D.W.; Analysis of Aldosterone by Gas-Liquid Chromatography, Anal. Biochem., Vol. 3, 1962, pp. 403 - 407.

   The 18-, 21 diacetate was produced which yielded a single symmetrical peak over a wide range of column temperatures. The eluted steroid was not identical to the injected diacetate.

2. Merits, I., Gas-Liquid Chromatography of Adrenal Cortical Steroid Hormones, Lipid Research, Vol. 3, 1962, pp. 126 - 127.

   The steroid was oxidized with periodic acid (15 hours) to produce the lactone of aldosterone which could be separated from the other steroids due to its insolubility in sodium bicarbonate. The procedure involved an ether extraction. The gamma lactone of aldosterone was stable under conditions of chromatography.

3. Woitz, H.H., Naukkarinen, I., Carr, H.E., Jr., Gas Chromatography of Aldosterone, Bio. Chim. et Biophys. Acta, Vol. 53, 1961, pp. 449 - 452.

   Aldosterone, acetylated at the 18-, 21-hydroxyl functions could be chromatographed in the vapor phase without decomposition. As little as $0.4 \mu g$ could be detected. Separation from cortisone was achieved. Chromatography required 15 - 20 minutes.

3. 3. 15 <u>17-Hydroxycorticosteroid (17-OH-CS) in blood serum or urine</u>

   A. Principles of present methodology

   1. Isotope dilution method for 17-OH-CS in blood serum

   The method most likely to be adaptable to space flight conditions is based on the capacity of 17-OH-CS to bind selectively to a blood serum alpha-1 globulin which has been called transcortin[8] or cortical steroid binding globulin (CBG).[1] The method is based primarily on two earlier reports.[4,5] Methods for the preparation of CBG have been described.[6,7] The method is based on the competition between isotopically labeled cortisol and cortisol endogenously present in 1 ml of blood serum for the binding sites on CBG. It is assumed that the isotopically labeled cortisol and the cortisol endogenously present in blood serum have an equal binding affinity for the sites on CBG. The number of isotopically labeled molecules which bind to the CBG will be a function of the total (labeled plus unlabeled) number of cortisol molecules present. It is only necessary then to add a known amount of isotopically labeled cortisol plus the cortisol present in 1 ml of blood serum to a standard quantity of CBG, remove the unbound cortisol and determine the radioactivity of the solution. By using known quantities of unlabeled cortisol in place of blood serum, a reference curve can be constructed from which cortisol concentrations in blood serum can be determined. Unbound cortisol is removed by adsorption on dextran coated charcoal.[5]

   2. Colorimetric method for 17-OH-CS in urine

   The steroids are extracted from urine as albumin-steroid complexes. In one method[9], egg or serum albumin is added to urine. The albumin is precipitated by saturating the solution with ammonium sulfate. The precipitated albumin-corticosteroid complexes are re-dissolved in water, and the albumin precipitated by organic solvents, leaving the free steroids in solution. Colorimetric

determination using 2, 4 dinitrophenylhydrazine or tetrazolium blue is done on the supernatant.

3. Enzymatic method for determination of 17-OH-CS in blood or urine

This method depends on reduction of the 20-oxo group of the corticosteroids by 20 β-hydroxycorticosteroid dehydrogenase and the simultaneous oxidation of stoichiometric amounts of reduced diphosphopyridine nucleotide (DPNH). As little as 0.25 microgram of steroid was determined with an accuracy of $\pm$ 5%. Both 17-hydroxy and 17-desoxy steroids are determined by the method, but by selective solvent extraction the method was made specific for the 17-OH-CS. Apparently a 20 β-hydroxycorticosteroid dehydrogenase specific for 17-OH-CS has not been described in the literature. If such an enzyme could be isolated, and used in the above procedure, a simple, highly specific procedure could be developed that does not require solvent extraction.

B. Suitability of present methodology to space flight conditions

1. Merit table

17-Hydroxycorticosteroids

Merit Parameters	Isotope dilution (Blood serum)	Colorimetric (Urine)	Enzymatic (Blood serum or urine)
1. Sensitivity	1 μg.	1 μg.	0.25 μg.
2. Sample size	1.0 ml.	0.3 ml. urine	0.3 ml. urine
3. Time required	1 hr.	45 min.	?
4. Reproducibility	10	5	8
5. Suitability for null gravity use	5	5	8
6. Overall safety	10	5	5
7. Nontoxic, noncaustic	10	3	3
8. Specificity	10	3	5
9. Insensitive to environmental changes	3	5	5
10. Ease in training personnel	5	5	5

17-Hydroxycorticosteroids – continued

Merit Parameters	Isotope dilution (Blood serum)	Colorimetric (Urine)	Enzymatic (Blood serum or urine)
11. Degree of separation required (10=none)	8	1	10
12. Minimal handling by analyst	5	4	6
13. Common use of analytic equipment	8	10	10
14. Nondestructive of sample	10	0	0
15. Figure of merit	57	10	27

2. Discussion

There are three major problems in the development of suitable methods of analysis of 17-OH-CS in urine. The first of these stems from the fact that the 17-OH-CS are excreted in conjugated froms. Most schemes of analysis begin with hydrolytic procedure, involving either acid or enzymatic hydrolysis. This is a time consuming step, requiring up to 24 hours. The second major problem lies in the fact that the methods for determination of these compounds are not specific, and accurate analytical data can only be obtained after multiple extractions with highly volatile organic solvents. The third problem with which we are faced with regard to the determinations of these compounds in urine results from the observation that although only three corticosteroids (cortisol, corticosterone and aldosterone) appear in blood serum, multiple metabolic products as well as several conjugated forms of the otherwise unchanged steroids are found in urine.

It is suggested that a knowledge of the 17-OH-CS concentrations in blood serum might be of more value than their rate of excretion. In view of the multiple problems concerned with their determination in urine, it is suggested that the isotope dilution method for determination of 17-OH-CS in blood serum be given prime consideration.

This method might also be adapted for the determination of unmetabolized 17-OH-CS in urine. This method probably would not detect the tetra-hydro derivatives of 17-OH-CS found in urine, however. Results obtained by the isotope dilution have been compared with other methods with very favorable results. [2]

C. Areas for research and development

1. Enzymatic

Search for 20-β-hydorxycorticosteroid dehydrogenase or other enzymes specific for 17-OH-CS.

2. Immunochemical methods

a. Investigate antigenicity of naturally occurring protein conjugates.

b. Prepare synthetic protein conjugates and investigate their antigenicity and specificity.

3. Vapor phase chromatography

Investigate stability and chromatographic behavior of trimethylsilyl ethers, acetates and other derivatives.

D. References

1. Doughaday, W.H.; Binding of Corticosteroids by Plasma Proteins, J. Clin. Invest., Vol. 37, 1958, pp. 511 - 518.

   Studied binding of corticosteroids and related hormones to transcortin by equilibrium dialysis, using $4-C^{14}$ labeled steroids.

2. Jones, J.A.; Mason, J.W.; A Critical Comparison of a Chromatographic and an Isotope Dilution Method of Plasma 17-Hydroxycorticosteroid Measurement, J. Clin. Endocrinol. and Metab., Vol. 26, 1966, pp. 1010 - 1111.

   Results obtained by the isotope dilution method compared favorably with those obtained by a conventional chromatographic method.

3. Margoff, C.O.; Weichselbaum, T.E.; A Method for the Enzymatic Determination of Corticosteroids in Extracts of Whole Blood, Plasma, and Urine, Steroids, Vol. 2, 1963, pp. 143 - 154.

   An enzymatic procedure based on reduction of the 20-oxo group of corticosteroids by 20 β-hydroxysteroid dehydrogenase and simul-

taneous oxidation of stoichrometric amounts of reduced DPN was applied to extracts from biological fluids. Solvent extraction was used to increase the specificity of the method.

4. Murphy, B. P.; Engelberg, W.; Pattee, C. J.; Simple Method for the Determination of Plasma Corticoids, J. Clin. Endocrinol. and Metab., Vol. 23, 1963, pp. 293 - 300.

   Describes a highly specific method for determining plasma corticoids using C^{14} labeled cortisol and corticosteroid binding globulin by a dialysis technique. One ml. of blood serum was used. A standard deviation of $\pm$ 1 μg. was obtained over a range of 1 to 10 μg/ 100 ml.

5. Nugent, C. A.; Mayes, D. M.; Plasma Corticosteroids Determined by Use of Corticosteroid Binding Globulin and Dextran Coated Charcoal, J. Clin. Endocrinol. and Metab., Vol. 26, 1966, p. 1116.

   Used dextran coated charcoal to adsorb cortisol which was not bound to corticosteroid binding globulin in an analytical technique using C^{14} labeled cortisol and 1 ml. of blood serum. Results compared favorably with older accepted chromatographic-fluorometric technique.

6. Seal, U. L.; Doe, R. P.; Corticosteroid-Binding Globulin: Species Distribution and Small Scale Purification, Endocrinol., Vol. 73, 1963, pp. 371 - 376. (CA 60, 2018a).

   Binding activity of CBG was studied and found to be tempera-dependent. A method of isolation and purification using hydroxy-lapatite is described.

7. Seal, U. S.; Doe, R. P.; Corticosteroid-Binding Globulin I Isolation from Plasma, J. Biol. Chem., Vol. 237, 1962, pp. 3136 - 3140.

   A procedure for preparation is described which results in a 1000-fold purification in 75% yield. The protein appeared homogenous. Its physicochemical properties are described.

8. Slaunwhite, W. R.; Sandberg, A. A.; Transcortin: A Corticosteroid Binding Protein of Plasma, J. Clin. Invest., Vol. 38, 1959, p. 3841.

   This paper represents the original description of transcortin. Its binding affinity for different corticosteroids is described.

9. Zumoff, B.; Bradlow, H. L.; Quantitative Extraction and Separa-

tion of Conjugated Steroid Metabolites from Human Urine, J. Clin. Endocrinol., Vol. 23, 1963, pp. 799 - 804.

Describes a procedure for quantitatively extracting conjugated steroid metabolites from urine using albumin. Conjugates were further studied by chromatography. It was concluded that the procedure does not introduce artifacts and that it yields quantitative results.

3.3.16 Catecholamines

A. Principles of present methodology[1, 2, 3, 4, 5, 6, 7, 8, 9, 10]

Biological, colorimetric and fluorometric techniques are available for the assay of catecholamines. Fluorometry seems to be the most simple and practical. Biological assays lack sensitivity and colorimetric tests lack both sensitivity and specificity. Fluorometry involves the isolation of the amines by an adsorption procedure, elution of the amines with acid, and then conversion to a fluorescent derivative. A symposium which appears in Pharmacological Reviews, Vol. 2, 1959, pp. 233 - 304, is an excellent review of the field and contains an extensive bibliography.

B. Suitability of present methodology to space flight conditions

1. Merit table

Catecholamines

Merit Parameters	Catecholamines in Urine Fluorescence	Catecholamines in Blood Fluorescence	"VMA" Colorimetric
1. Sensitivity	very good	very good	good
2. Sample size	24 hr. sample	13 ml. plasma	1.0 ml. urine
3. Time required	approx. 2 hrs.	approx. 2 hrs.	8
4. Reproducibility	4	3	8
5. Suitability for null gravity use	-	-	-
6. Overall safety	8	8	8
7. Nontoxic, noncaustic reagents	8	8	8
8. Specificity	5	5	4
9. Insensitive to environmental changes	8	8	10
10. Ease in training personnel	2	1	4
11. Degree of separation required (10=none)	1	1	3
12. Minimal handling by analyst	2	1	5

Merit Parameters	Catecholamines in Urine Fluorescence	Catecholamines in Blood Fluorescence	"VMA" Colorimetric
Catecholamines - continued			
13. Common use of analytic equipment	8	8	10
14. Nondestructive of sample	6	4	8
15. Merit range	10-19	7-14	23-46
16. Mean figure of merit	14	10	34

2. Discussion

As is true of many analyses of substances from biological sources it is necessary to perform certain isolation and purification steps. Currently, the use of alumina as an adsorbent results in recoveries of about 85%. This many be due in part to losses at neutral pH. It may be better to use a cation exchange resin such as Dowex-50, which will adsorb the catechol amines under acid conditions and allow the removal of the amines under stronger acid conditions. All the methods involve a large number of manipulations and consequently the number of determinations which can be accomplished is limited. Some estimate 5- 8 per day.

C. Areas for research and development

A simplified approach to this problem might be the combination of paper chromatographic separation by adsorption followed by direct fluorometry on paper using equipment like the chromatogram door supplied with the Turner Fluorometer.

Some investigators have chosen to use the urinary excretion of 3-methoxy-4-hydroxymandelic acid (VMA) as an index of catechol amine metabolism and excretion. It can be determined by either chromatography or solvent extraction followed by colorimetric determination.[4,5,8] Simple modifications of methods for VMA should be

possible which would enable the determination under space flight conditions.

D. References

1. Brunjes, Shammon; Wybenga, D.; Differential Fluorimetry in Catechol Amine Determination: A Simplified Method of Calculation. Clin. Chem., Vol. 9, 1963, pp. 626 - 630.

   A method for solving two simultaneous equations for the differential fluorimetry of epinephrine and norepinephrine using internal standards for urine analysis.

2. Callingham, B. A.; Cass, R.; The Determination of Catechol Amines in Biological Materials. West European Symp. Clin. Chem., Vol. 2, 1963, pp. 19 - 30.

   For the extraction and purification of the catechol amines in plasma and urine adsorption and ion-exchange (Dowex 50) are used. To obtain sensitivity with specificity, fluorimetric methods are necessary.

3. Cardona, R.; Soehring, K.; Thin-layer Chromatographic Detection of Micro Amounts of Catechol Amines and their Derivatives, Med. Exptl., Vol. 10, 1964, pp. 251 - 257.

   Method for preparation of plates and the separation of 0.05 gamma of catecholamines and their derivatives is described.

4. Connelian, T. P.; Godfrey, J. M.; Routine Determination of Urinary 4-hydroxy-3-methoxymandelic Acid, Clin. Chem. Acta, Vol. 9, 1964, pp. 410 - 412.

   Modification of the method of Pisano, et al. (Chemical Abstracts, Vol. 57, p. 1147g) so that critical pipetting operations were reduced to 2 by using a single set of 100 ml. cylindrical short stemmed separate funnels. The new technique increased overall recovery, eliminated the need for microcuvets and reduced analysis time.

5. Crymble, G.; A Comparison and Evaluation of Colorimetric Procedures for 3-methoxy-4-hydroxymandelic Acid, Can. J. Med. Technol., Vol. 26, 1964, pp. 188 - 197.

   The extraction of the title compound from acidified urine into ethyl acetate and reextraction into aqueous $K_2 CO_3$ was found essential. The adsorption of Florisil approached the extraction into

ethyl acetate. The absorption curves of processed urines did not approach those of pure title compound and many phenolic acids may be measured with the title compound.

6. Kawai, S.; Nagatsu, T.; Imanari, T.; Gas Chromatography of Catecholamines and Related Compounds, Vol. 14, 1966, (Eng.), pp. 618 - 621.

   A satisfactory separation of epinephrine, norepinephrine, doapmine, metanephrine and normetanephrine was achieved through trimethylsilylation with hexamethyldisilazane followed by condensation with 2-penatanone.

7. Kirschner, N.; Goodall, McC.; Separation of Adrenaline, Noradrenaline and Hydroxytyramine by Ion-Exchange Chromatography, J. Biol. Chem., Vol. 226, pp. 207 - 212.

   A procedure is described for the separation of adrenaline, noradrenaline and hydroxytyramine by use of Amberlite IRC-50. Recoveries from pure solutions ranged from 85 - 97%.

8. Mahler, D. J.; Humoller, F. L.; Comparison of Methods for Determining Catechol Amines and 3-methoxy-4-hydroxymandelic Acid in Urine, Clin. Chem., Vol. 8, 1962, pp. 47 - 55.

   Methods for the evaluation of pheochromocytoma were compared. The results obtained by bioassay, fluorimetry, and spectrophotometry in normal subjects are reported.

9. Mattok, G. L.; Wilson, D. L.; Separation of Catechol Amines and Metanephrine and Nor-Metanephrine Using a Weak Cation-Exchange Resin, Biochem., Vol. 11, 1965, pp. 575 - 579.

   The title compounds were adsorbed on Amberlite IRC-50 and the catechol amines eluted by a boric acid solution and metanephrine and normetanephrine by $H_2 SO_4$.

10. Ritzel, G.; Hunzinger, W. A.; Determination of Catechol Amines in Urine, Klin. Wochschr., Vol. 41, 1963, pp. 419 - 423.

    The catechol amines were oxidized to adrenochrome and reduced to the corresponding trihyroxyindoles and determined fluorimetrically. Normal data for the excretion of adrenaline, noradrenaline and vanilmandelic acid are listed.

3. 3. 17 Serotonin

A. Principles of present methodology

The unique fluorescence charactheristics of serotonin (5-hydroxy-tryptamine) make possible its direct determination in blood after removal of protein. This method lacks specificity in that all 5-hydroxy-indoles have the same general fluorescence characteristics. Serotonin, however, is the only hydroxyindole found in tissues. In the normal subject only 0.05 - 0.2 micrograms/ml. are found which makes direct measurement impossible.[2,6]

A number of methods for the assay of 5-HIAA, a metabolite of serotonin, are available in the literature.[1,3,4,5] A semiquantitative assay is based on a color reaction with 1-nitroso-2-naphthol.[4] This reaction can also be used in a more quantitative extraction procedure.[5]

B. Suitability of present methodology to space flight conditions

1. Merit table

Serotonin

Merit Parameter	Serotonin	5-HIAA
1. Sensitivity	< 0.035 μg/ml.	40 μg/1 (urine)
2. Sample size	?	3
3. Time required	1 hour	20 minutes
4. Reproducibility	2	3
5. Suitability for null gravity use	0	0
6. Overall safety	1	1
7. Nontoxic reagents	2	3
8. Specificity	2	8
9. Insensitive to environmental changes	0	0
10. Ease in training personnel	0	5
11. Degree of separation required (10=none)	0	3
12. Minimal handling by analyst	1	3
13. Common use of analytic equipment	5	5

Serotonin - continued		
Merit Parameters	Serotonin	5-HIAA
14. Nondestructive of sample	0	0
15. Figure of merit	1	4

2. Discussion

The simple test reproted by Sjoerdsma[6] and Elliott[4] should have direct application. Since this determination has generally been used for the diagnosis of carcinoid tumors of the intestine, however, its significance in evaluation of the effect of space flight on man appears doubtful. The concentration of 5-HIAA in normal urine is not detectable by this method.

C. Areas for research and development

Strum[6] has suggested that amounts of 5-hydroxytryptamine normally found in plasma can be quantitated by paper electrophoresis of deproteinized serum, treatment with Ehrlich's reagent, direct elution and measurement of absorption at 275 mμ. The method recently reported by Contractor[1] seems to hold the most promise for the simple determination of 5-HIAA. The 5-HIAA is selectively absorbed on Sephadex G-10[*], and interfering fluorophores are then leached out and the 5-HIAA is eluted with NH_4OH and determined with spectrophotofluorimetry.

D. Comments

It seems that several of these methods could be applicable immediately, if methods can be developed for handling liquids and volatile solvents in a space environment.

E. References

1. Contractor, S. F.; A Rapid Quantitative Method for the Estimation of 5-hydroxyindoleacetic Acid in Human Urine, Biochem. Pharmacol., Vol. 15, 1966, pp. 1701 - 1706.

   An adsorption separation followed by elution and spectrophotofluorometry.

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[*] Registered trademark of Pharmacia, Inc., Stockholm, Sweden.

2. Crawford, N.; Rudd, B. T.; A Spectrophotofluorometric Method for the Determination of Serotonin in Plasma, Clin. Chim. Acta, Vol. 7, 1962, pp. 144 - 121.

   A method for analysis of platelets or platelet rich plasma. Ether extraction is used or purify the serotonin before analysis.

3. Deux, C.; Delauneus, B.; Critical Testing of Biochemical Methods for the Investigation of Serotonin Metabolism, Presse. Med., Vol. 72, 1964, pp. 2925 - 2930.

   Methods for the determination of 5-HIAA in urine are reviewed. 19 references.

4. Elliott, H. C.; Casey, A. E.; Experience With a Simple Screening Test for Serotonin, Sou. Med. J., Vol. 51, 1958, pp. 836 - 840.

   A modified colorimetric procedure for 5-HIAA was used to screen 4,517 urine samples from routine hospital admissions.

5. Kirberger, E.; Significance and Method of Detection of Increased Urinary Excretion of 5-HIAA, Deut. Med. Wochschr., Vol. 91, 1966, pp. 2128 - 2130.

   A modification of the method of Sjoerdsma (see 6.)

6. Sjoerdsma, A.; Weissbach, H.; Udenfriend, S.; Simple Test for the Diagnosis of Metastic Carcinoid, J.A.M.A., Vol. 159, 1955, pp. 397 - 399.

   A color reaction for semiquantitative estimation of 5-HIAA.

3.3.18 Thyroxine, thyroxine binding prealbumin, protein bound iodine

Measurement of the thyroid hormones in the serum has been the most valuable method of assaying thyroid function. Indirect measurement by determination of protein-bound iodine[3,4,6,12] has been the method of choice for some years. It can be demonstrated that the PBI level is directly proportional to the serum thyroxine level. Recently it has been possible to develop highly specific assay for thyroxine[5,7,8,9] based upon work with thyroxine-binding globulin.

A. Principles of present methodology

The Barker method for determination of PBI depends upon the steps of removal of inorganic iodide from serum, conversion of organic iodine into inorganic iodide, and the determination of the iodide formed by measuring its catalytic effect on the reduction of Ce^{+4} to Ce^{+3} by As^{+3}.

The thyroxine-binding globulin[2,11,13] (TBG) is generally used as a measure of the thyroid hormone transport system. The bound thyroid hormones are in continuous equilibrium with a small amount of circulating free hormone. Ultimately these plasma carrier proteins exchange thyroid hormone with binding proteins of the tissues, and it is probably the free moiety of thyroid hormone which is physiologically active. Thyroxine (T-4) is bound preferentially to triiodothyronine (T-3), and free T-4 will replace bound T-3. Free T-3, however, will not replace bound T-4. This equilibrium and preferential binding of T-4 over T-3 is the basis of two thyroid function tests involving thyroid hormone transport. TBG measures the total thyroxine binding capacity and indicates the quantity of TBG in circulation. The procedure is performed by the addition of excess of I^{131} labeled thyroxine to the subjects serum, followed by electrophoretic separation of the TBG inter-alpha band.

Assay for total thyroxine[7,10] depends on the equilibrium previously mentioned. In the test, a pooled serum is saturated with I^{131} thyroxine

so that an equilibrium is established between free and bound radio-active thyroxine. A measured amount of this serum is added to an alcholic extract of the patient's serum. This extract contains the patient's total thyroxine. The amount of thyroxine in this extract will upset the equilibrium in the serum and establish a new equilibrium; dependent upon the amount of thyroxine in the extract. After passing the serum solution through a sephadex gel, the amount of bound thyroxine is measured and the patient's total thyroxine level is calculated by comparing the radioactivity of the serum treated with the extract from the subject's serum with serums treated with known amounts of pure thyroxine.

B. Suitability of present methodology to space flight conditions

1. Merit table

Thyroid Function

Merit Parameters	PBI	Total Thyroxine	TBG
1. Sensitivity	Excellent	Good	Unknown
2. Sample size	All would require several ml. of subject's serum.		
3. Time required	2 hrs.	Unknown	Unknown
4. Reproducibility	9	5	5
5. Suitability for null gravity use	10	8	8
6. Overall safety	2	8	8
7. Nontoxic, noncaustic reagents	0	9	9
8. Specificity	7	6	6
9. Insensitive to environmental changes	-	-	-
10. Ease in training personnel	2	4	3
11. Degree of separation required (10=none)	4	3	2
12. Minimal handling by analyst	1	2	2
13. Common use of analytic equipment	*	*	**
14. Nondestructive of sample	0	3	3
15. Merit range	11-22	18-26	17-25
16. Mean figure of merit	18	22	21

* Not weighted so that merit ratings comparable. (Photometry)
** Depends on availability of adequate counting equipment.

2. Discussion

The two methods currently used for PBI chemical determination (Chaney and Barker) are quite difficult technically. They involve, among other difficulties, either wet or dry ashing of a serum sample, and the use of very strong sulfuric acid solution. It does not seem likely that such procedures could be accomplished in space. Other hormone transport measurements involve the use of I^{131} labeled thyroxine and triiodothyronine and therefore fairly sophisticated counting equipment. This obstacle would not appear to be insurmountable however. The most likely approach will be to simplify in some way the techniques of electrophoretic separation and isotope counting.

C. Areas for research and development

It would seem that in the current state of knowledge concerning thyroid physiology the determination of either total thyroxine[7] or free thyroxine[5,7] would be the method of choice for development. Determination of free thyroxine would be technically much more complex simply because it is present in serum in very small amounts (3 - 6 mµg/100 ml.)

It may also be possible that the electron probe x-ray analyzer can be applied after a preliminary separation of organic and inorganic iodine.[1]

D. References

1. Bowen, H. J. M.: Cawse, P. A.; The Determination of Inorganic Elements in Biological Tissue by Activation Analysis, U. K. At. Energy Authority Rept. AERE-R 4309, 1963, p. 39.

   Neutron activation was used for determining Ca, Cl, Cu, I, Mg, etc., in biological tissue. A very selective and sensitive analysis achieved by chemical separation before counting. Accuracy $\pm$ 5%.

2. Braverman, L. E.; Dawber, N. A.; Ingbar, S. H.; Binding of Thyroid Hormones in Sera of Normal Subjects of Varying Ages,

J. Clin. Invest., Vol. 45, 1966, pp. 1273 - 1279.

The average endogenous thyroxine (T_4) distribution on T_4-binding globulin (TBG), albumin, and prealbumin (TBPA) was determined for age groups from 2 years to 70 years in 10-year intervals. In the group 31 - 40 the distribution was TBG 39.5%, albumin 15.8%, TBPA 44.6%.

3. Gardiner, E.; Burns, A.; Micromethod for the Rapid Determination of Serum Protein-Bound Iodine and Total Serum Iodine, Clin. Chem., Vol. 10, 1964, pp. 1137 - 1146.

A modification of the colorimetric chloric acid method which requires less than 0.5 ml serum for duplicate determination.

4. Hellauer, H.; Significance and Application of the PBI Determination, Wien, Klin, Wochschr., Vol. 78, 1966, pp. 553 - 556.

Free thyroxine in serum calculated from the degree of discussion of the protein complex of I^{131} labeled hormone and PBI. Eleven procedures for determining PBI are compared and 6 for determining total thyroid hormone binding proteins.

5. Henry, R.J.; Golub, O.J.; Determination of the Free Thyroxine Content of Serum, J. Clin. Endocrinol. Metab., Vol. 24, 1964, pp. 486 - 495.

After equilibration of thyroxine ^{131}I with serum protein bound iodine the specific activity of the free thyroxine is the same as that of protein bound thyroxine. Free thyroxine was separated from bound by Sephadex G-25. Free thyroxine was calculated from net count rate of Sephadex bound ^{131}I, net count rate of protein-bound ^{131}I, and serum thyroxine I.

6. Lerner, S.R.; Iodinated Amino Acid Chromatography on Polystyrene Resins. Arch. Biochem. Biophys., Vol. 103, 1963, pp. 36 - 41.

Iodinated amino acids eluted from Dowex-50-X4 using formamide buffers.

7. Murphy, B.P.; Pattee, C.J.; Determination of Thyroxine Using the Property of Protein Binding. J. Clin. Endocrinol. Metab., Vol. 24, 1964, pp. 187 - 196.

Use of I^{131} labeled thyroxine to determine protein binding

globulin and measurement of equilibrium displacement as more T_4 is added.

8. Nakajima, H.; Kuramochi, M.; Horiguchi, T.; A New Simple Method for the Determination of Thyroxine in Serum, Nippon Naibumpi Gakkai Zasshi, Vol. 40, 1965, pp. 1303 - 1307.

   Method based on use of pooled serum of known triiodothyronine binding capacity. Amberlite IRA-400 used to separate bound and unbound I. Linear relation established between amounts of thyroxine in serum and resin bound I^{131} thyroxine.

9. Oppenheimer, J.H.; Martinez, M.; Berstein, G.; Determination of the Maximal Binding Capacity and Protein Concentration of Thyroxine Binding Prealbumin in Human Serum, J. Lab. Clin. Med., Vol. 67, 1966, pp. 500 - 509.

   An improved method for determining the maximum binding capacity of TBPA using paper electrophoresis. Mean level in a group of normal adults was a maximum binding capacity of 274 gamma/100 ml.

10. Pileggi, V.J.; Lee, N.D.; Golub, O.J.; Determination of Iodine Compounds in Serum. I. Serum Thyroxine in the Presence of Some Iodine Contaminants, J. Clin. Endocrinol. and Metabolism, Vol. 21, 1961, pp. 1272 - 1279.

   A Dowex-1 column is used to isolate more than 80% of added I^{131} in the first 10.0 ml of eluate.

11. Rich, C.; Bearn, A.G.; Localization of the Thyroxine-Binding Protein of Serum by Starch Gel Electrophoresis, Endocrinology, Vol. 62, 1958, pp. 687 - 689.

   Thyroxine binding protein migrated ahead of albumin in a sharp band.

12. Schorn, H.; Winkler, C.; Thin Layer Chromatographic Analysis of Thyroid Hormones, J. Chromatography, Vol. 15, 1965, pp. 69 - 75.

   Triiodothyronine and thyroxine were separated in 2 hrs. by thin layer chromatography on silica gel G. Use of thin-layer chromatography in a clinical study of hyperthyroidism is discussed.

13. Tata, J.R.; Purification of Thyroxine Binding Globulin (TBG) and Thyroxine Binding Prealbumin (TBPA), Clin. Chim. Acta, Vol. 6, 1961, pp. 819 - 832.

Methods described for isolating these two major thyroxine binding proteins from human serum. Fractions were characterized by ultracentrifugation, immunoelectrophoresis, gel diffusion and zone electrophoresis.

3. 3. 19 Amino nitrogen

To measure accurately the amino acid concentration of a biological sample it is first necessary to isolate and measure each of the twenty or so compounds and quantitate each individually. This is required because there are large differences in the molecular weights, reactivities, and abundances of the various amino acids. To adequately isolate and measure the various amino acids would require equipment and techniques which appear to be too complex and cumbersome for the current application.

A. Principles of present methodology

Methods commonly used to measure amino nitrogen generally employ colorimetric techniques which assay total amino nitrogen as compared with a selected amino acid standard. The three most popular techniques employ protein-free solutions.

1.   One method depends upon the color produced by reaction of amino compounds with ninhydrin.[4] This procedure yields values greater than other techniques and thus there is some question about the specificity.

2.   The method of choice is one utilizing the color produced by reaction of amino acids with β-naphthoquinonesulfonate.[3,5]

3.   The third procedure involves the addition of cupric phosphate to the sample. Copper forms soluble complexes with amino groups and the measurement of copper in the supernatant is then a measure of amino nitrogen. To obtain satisfactory results with blood samples it is necessary to isolate the amino compounds with an ion-exchange resin prior to equilibration with the cupric phosphate. The numerous manipulations required for this procedure eliminate it from further consideration.

B. Suitability of present methodology to space flight conditions

1. Merit table

Amino Nitrogen

Merit Parameters	Method 1	Method 2
1. Sensitivity	Good	Good
2. Sample size	300 µl	500 µl
3. Time required	45 min	45 min
4. Reproducibility	10	9
5. Suitability for null gravity use	-	-
6. Overall safety	0	6
7. Nontoxic reagent	0	1
8. Noncaustic reagent	1	1
9. Specificity	-	7
10. Applicability	6	6
11. Reagent volatility	0	4
12. Insensitive to environmental changes	1	1
13. Ease in training personnel	7	6
14. Minimal handling by analyst	5	4
15. Common use of analytic equipment	10	10
16. Merit range	5-17	13-26
17. Mean figure of merit	10	20

2. Discussion

All three of the methods presented for the measurement of amino nitrogen require equipment for the preparation of protein free solutions (centrifuge, dialysis device, etc.) and colorimeter.

C. Areas for research and development

Perhaps the most fruitful area for exploration would be gas-liquid chromatography. This technique can now be used to afford complete separation and quantitation of the naturally occurring amino acids. Many support media are stable and many times reusable. Probably the greatest problem would be encountered in preparing derivatives; however, the operation could undoubtedly be largely or entirely automated. The instrument output would be best interfaced with data

processing equipment to provide digital values for the individual constituents.

Another approach would be to form derivatives with the amino acids which would have characteristic optical properties. Such a compound currently enjoying considerable popularity is 1-dimethyl-aminonaphthalene-5-sulfonyl halide, often disguised as the acronym dansyl. This compound has had frequent application for N-terminal end-group analyses of proteins[1] and for "tagging" amino acids.[2] Another possible compound for derivatizing amino acids is 2-chloro-3, 5-dinitropyridine. This substance can be used to form derivatives in aqueous solutions under very mild conditions.

A method employing amino acid oxidase might also be explored. L-Amino acid oxidase catalyzes the oxidative deamination of most of the amino acids with the release of ammonia. The ammonia produced could be estimated using one of the methods discussed under Blood Urea Nitrogen. One great disadvantage of this approach is the great variation in reaction rates for the different amino acids. This aspect might be resolved by using long incubation times to approach complete deamination for all the amino acids.

It may also be possible to employ bacterial amino acid auxo-trophs to devise a simple microbiologic assay.

D. References

1. Crombrugghe, B.; Edelhoch, H.; The Properties of Thyroglobulin XIV, Biochem., Vol. 5, 1966, pp. 2238 - 2245.

   Used 1-dimethylaminonaphthalene-5-sulfone derivatives of thyroglobulin to study structure.

2. Deranleau, D. A.; Neurath, H.; The Combination of Chymotrypsin and Chymotrypsinogen with Fluorescent Substrate and Inhibitors for Chymotrypsin, Biochem., Vol. 5, 1966, pp. 1413 - 1425.

   Employed amino acid ester derivatives of 1-dimethylamino-naphthalene-5-sulfone to study interaction of enzyme and substrate.

3.3.20 <u>Blood urea nitrogen (BUN)</u>

    A. Principles of present methodology

        Most clinical measurements of urea employ either a direct chromo-
genic reaction with diacetyl or the action of urease followed by a tech-
nique to quantitate the ammonia produced.

    1. Diacetyl

      a.   The use of the diacetyl reaction to measure urea colori-
metrically has become popular with continuous flow automated
equipment. The reaction is quite sensitive and difficult to con-
trol when employed in manual procedures; however, with auto-
mated equipment, where conditions are very reproducible, the
method is quite good.

          The reaction is not specific in that apparently most ureido-
compounds will react to yield chromogens. Other disadvantages
are that the color produced fades quickly, is photosensitive,
does not show a linear relationship between concentration of
urea and absorbance, and the heating time required for maximum
color development depends upon the urea concentration.[5]

      b.   Diacetyl can also be used in a fluorometric procedure.[7]
When urea and diacetylmonoxime are heated with sulfuric acid
and irradiated at 380 mμ the reaction products show fluores-
cence at 420 and 520 mμ.

    2. Urease

        The utilization of urease in the measurement of urea is a well
established procedure. Urease is extremely specific, has great
activity, is easily prepared, and is stable for long periods in cer-
tain solutions or when lyophilized. Most methods that utilize urease
measure the ammonia liberated by nesslerization or by the Berthelot[3]
reaction.

      a.   Nessler's reagent, $(KI)_2 HgI$, is easily prepared and very
stable. The colored product of nesslerization is colloidal and

generally requires a protective colloid for photometry.

b. The Berthelot reaction is about ten times more sensitive to ammonia than nesslerization and the colored product is soluable and quite stable. All of the reagents required are stable except for the hypohalite (usually hypochlorite).

3. Xanthydrol

Xanthydrol is a very specific reagent for urea and can be employed in several ways.

a. In a turbidimetric method[6] a sample of serum in acetic acid is mixed with an alcoholic solution of xanthydrol. After 30 minutes the turbidity is measured at 546 mμ. Xanthydrol can also be employed in colorimetric procedures.

b. In one procedure[1] a protein-free filtrate of blood is reacted with xanthydrol. The dixanthydryl urea product is collected by filtration, washed, and dissolved in 50% H_2SO_4. The absorbance of the yellow dixanthydryl urea solution is then measured and compared with similarly treated standard urea solutions.

c. Another colorimetric procedure involves the reaction of the separated dixanthydryl urea with hydrogen peroxide and phenol reagent.[2, 8]

4. Coulometry can also be used in the analyses of urea, following the conversion to ammonia. However, the ammonia must be isolated from interfering substances prior to oxidation. Isothermic distillation (diffusion) has been employed for the separation of the ammonia.[4]

5. There are commercially available "dipsticks" which can be used for very crude evaluations of urea concentrations. It is possible that these could be modified to provide better quantitation. These devices will not be considered further because they are,

at best, semi-quantitative.

B. Suitability of present methodology to space flight conditions

   1. Merit table

Blood in Urea Nitrogen

Merit Parameters	1a	1b	2a	2b	3a	3b	3c	4
1. Sensitivity	good	very good	very good	excel-lent	good	fair	-	good
2. Sample size	50 µl	10 µl	10 µl	20 µl	100 µl	500 µl	-	50 µl
3. Time required	30 min	40 min	20 min	70 min	40 min	60 min	-	150 min
4. Reproducibility	8	-	10	8	-	-	-	-
5. Suitability to null gravity	-	-	-	-	-	-	-	-
6. Overall safety	2	2	6	5	8	2	2	8
7. Nontoxic reagents	2	2	5	3	4	2	3	6
8. Noncaustic reagents	1	1	8	6	5	1	3	8
9. Specificity	6	7	9	9	9	9	9	9
10. Applicability	5	5	8	6	9	5	5	5
11. Reagent volatility	1	1	10	3	3	10	9	10
12. Sensitivity to environ-mental changes	10	10	10	2	10	10	10	10
13. Ease in training personnel	8	8	8	7	9	8	7	6
14. Manipulations	5	5	8	7	10	1	2	1
15. Common use of analytic equipment	10	7	10	10	8	10	10	1
16. Merit range	10-21	0-27	32-65	16-33	0-62	0-33	0-35	0-60
17. Mean figure of merit	16	10	48	24	23	12	13	22

   2. Discussion

     It would seem that the best procedure for immediate use would be method 2a. The method enjoys the specificity of urease and the reagents are stable. The Nessler's reagent is toxic, but it is not volatile and not very caustic. Quantitation is by photometry

(colorimetry), measuring technique which will undoubtedly be common for many other procedures.

The technique consists of adding the serum sample to lyophilized urease. The buffering capacity of the serum precludes the use of additional buffer. By using very active urease, incubation at room temperature for 5 - 10 minutes is adequate for complete urea hydrolysis. Following deproteinization the protein-free solution is reacted with Nessler's reagent. The absorbance of the resulting colored solution, stabilized by a protective colloid (usually gum ghatti) is measured at around 400 mμ. The absorbance of the sample is compared with a similarly treated standard. One good aspect of nesslerization is that if the absorbance is too great at lower wavelengths, valid measurements can be made at longer wavelengths (450 - 550 mμ) where the extinction coefficient of Nessler's compound is less.

The approaches which would appear most promising for future application would probably be those discussed in paragraphs C.2, C.3, and C.1 in that order. If the substance used to prepare the urea derivative was such that it did not interfere with subsequent determinations, the approach would be essentially non-destructive.

Conductivity measurements, discussed in paragraph C.3, should involve simple equipment and following the measurement, the sample should be suitable for other determinations.

The electrometric techniques seem to present more problems in that the alkalinization for ammonia release or acidification for carbon dioxide liberation might interfere with subsequent measurements with the sample. Also the isolation of the ammonia or carbon dioxide would appear to be difficult under the conditions of the present application.

Equipment

a. Method la

A centrifuge (protein free solution), heating bath and colorimeter. Reagents stable.

b. Method lb

A centrifuge (protein free solution), heating bath and fluorimeter. Reagents stable.

c. Method 2a

Centrifuge and colorimeter. Reagents stable.

d. Method 2b

Colorimeter only required. Hypochlorite reagent unstable for prolonged periods.

e. Method 3a

Colorimeter or nephelometer (fluorimeter) required.

f. Method 3b

Centrifuge and colorimeter.

g. Method 3c

Centrifuge and colorimeter.

h. Method 4

Coulometer with amperometric detector and isothermic distillation apparatus or similar device (ion-exchange resin system or aeration system are possibilities).

C. Areas for research and development

1. The enzymic hydrolysis of urea with urease produces ammonia and carbonic acids. If either of these two products could be isolated and introduced into solutions with known buffer capacities then pH measurements could conceivably serve for quantitation.

Ammonia can be readily separated from solutions by alkalinization and distillation. Isothermic distillation, where the ammonia is collected as non-volatile ammonium ion, has enjoyed

extensive application in clinical chemistry. The major problem with this technique and the present application is that of keeping the solutions (sample and collecting solution) separated. This might be done with membranes which would be permeable to ammonia and perhaps vaporized water, but would be impermeable to the sample solution. To reduce distillation time the operation might be done with very thin films with large surface areas.

Perhaps more promising would be a method to measure the carbon dioxide produced. Carbon dioxide can be distilled from samples by acidifying the sample and collecting the volatile gas in alkali. Another approach would be to use a membrane permeable to carbon dioxide to separate the sample and the buffer solution, such as described below for the measurement of serum bicarbonate. The increase in pCO_2 from initial to final would then be related to the amount of urea originally present. Aeration could also be used in place of distillation, but a solution barrier (hydrophobic membrane permeable to gases) would be required to separate sample and receiving solutions.

2. The optical properties of urea derivatives might also provide simple specific methods of quantitation. An obvious example would be to use xanthydrol and measure the absorbance and/or fluorescence at characteristic wavelengths. Derivatives formed by reaction with phthalyl halide or p-nitrobenzyl halide might also be worth investigating.

3. Conductivity measurements before and after hydrolysis of urea by urease would appear to be a meaningful approach. Conductivity can be measured on a very small sample, and except for the addition of urease and the destruction of urea, the sample suffers no alterations. It would seem that an initial conductivity measurement (blank) would not be required so long as several values of conductivity were made during hydrolysis so that an initial conductivity

value could be estimated by extrapolation. The increase in conductivity as a result of the formation of ammonium carbonate from urea would then be a measure of urea concentration.

D. References

1. Beattie, F.; Micro-method for the Colorimetric Determination of Urea in Blood, Biochem., J., Vol. 22, 1928, pp. 711 - 712.

   Describes a colorimetric technique for urea using xanthydrol.

2. Carr, J.J.; Alkaline and Acid Phosphatase, Standard Methods of Clinical Chemistry, Vol. 1, 1953, pp. 75 - 83.

   Describes the preparation of phenol reagent as used in phosphatase assay.

3. Chaney, A.L.; Marbach, E.P.; Modified Reagents for Determination of Urea and Ammonia, Clin. Chem., Vol. 8, 1962, p. 131.

   Combined reagents for Berthelot reaction and found they were quite stable when stored under proper conditions.

4. Christian, G.D.; Knoblock. E.C.; Purdy, W.C.; A Coulometric Determination of Urea Nitrogen in Blood and Urine, Clin. Chem., Vol. 11, 1965, pp. 700 - 707.

   Presents a coulometric analysis for urea, but requires isolation of ammonia.

5. Henry, R.J.; Clinical Chemistry, Principles and Technics, Harper and Row Publishers, New York, 1964, pp. 263 - 264.

   A book devoted entirely to the principles and techniques of chemistry. The author generally discusses several methods in some detail and then selects one or two and describes them in great detail. Provides values of accuracy, precision and normal values for selected procedures.

6. Kachani, Z.F.Ch.; Simplified Determination of Urea in Serum, Aergtl. Lab., Vol. 9, 1963, pp. 81 - 86.

   Describes a direct turbidimetric method for measuring urea with xanthydrol.

7. McCleskey, J.E.; Fluorometric Method for the Determination of

Urea in Blood, Anal. Chem., Vol. 36, 1964, pp. 1646 - 1648.

8. Yoshimatou, S.; A New Colorimetric Urea Method with 0. 1 ml. of Blood, Tohoku J. Exptl. Med., Vol. 13, 1929, pp. 1 - 5.

   Presents a colorimetric procedure for urea using xanthydrol, hydrogen peroxide and phenol reagent.

3.3.21 Non-protein nitrogen (NPN) and total nitrogen

Essentially all specificity is lost when these two groups of nitrogenous compounds are to be analyzed. The only aspect in common for the several different materials assayed in these two groups is that they all contain nitrogen. In the instance of NPN it is the total nitrogen in protein-free blood solutions and for total nitrogen of urine it is all the nitrogenous urinary components. The nitrogen in both cases is in various forms, including ammonia, amines, amides and as constituents of heterocyclic rings. To assay nitrogen in the various forms it seems necessary to convert it first to a common form. The technique suggested is conversion to ammonia, a compound readily assayed.

A. Principle of present methodology

1. NPN

The sample of serum plasma or whole blood is treated with an alkaloidal reagent (usually trichloroacetic or tungstic acid) to prepare a protein-free solution. The filtrate (supernatant solution of dialyzate) is then treated as urine for total nitrogen as described below.

2. Total nitrogen

The classical and probably the best technique for converting nitrogen in biological samples to ammonia is a modification of the Kjeldahl technique.[1] An aliquot of the protein-free solution or urine is subjected to wet acid digestion. Sulfuric and selenious acids are generally the main reagents, although other reagents, such as syrupy orthophosphoric acid, are often added to raise the boiling point and thus shorten digestion time. The ammonium ions produced can be quantitated in various ways, but for convenience and economy nesslerization appears most suitable as described above for the analysis of urea.

B. Suitability of present methodology to space flight conditions

1. Merit table

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Non-protein Nitrogen and Total Nitrogen

Merit Parameters	NPN	Total Nitrogen
1. Sensitivity	Good	Good
2. Sample size	100 µl	10 µl
3. Time required	60 min	60 min
4. Reproducibility	9	9
5. Suitability to null gravity use	-	-
6. Overall safety	0	0
7. Nontoxic reagents	1	1
8. Noncaustic reagents	1	1
9. Specificity	9	9
10. Applicability	0	0
11. Reagent volatility	1	1
12. Sensitivity to environmental changes	8	8
13. Analyst training	4	3
14. Manipulation	3	3
15. Common use of analytic equipment	10	10
16. Merit range	7-15	7-14
17. Mean figure of merit	11	11

2. Discussion

As indicated previously it seems necessary to convert all the nitrogen to a state where a single technique can be applied for quantitation. All existing methods for the degradation of organic materials require either wet digestion or combustion; processes which do not appear entirely desirable under the conditions specified.

NPN and total nitrogen determinations under the prescribed conditions do not appear feasible. The wet acid digestion requires the continuous removal of volatile components and a failure in this removal could lead to serious environmental contamination.

Measurements of NPN for evaluations of kidney function can be largely, and often better, replaced by BUN assays. Assessment of metabolic states, which can perhaps be measured in part by NPN, can probably be done as well with estimations of blood amino acid nitrogen.

There is apparently no substitute for the measurement of urinary nitrogen. Over an extended period the nitrogen balance can be approximated from a knowledge of the nitrogen intake and body weight. Also urine urea can be used as a crude measure of nitrogen balance in that normally the majority of the urinary nitrogen occurs as carbamide.

<center>Conditions and Equipment</center>

NPN assay requires equipment to prepare protein-free solutions. Both procedures need digestion equipment with provision for the removal of toxic fumes, and a colorimeter.

C. Areas for research and development

(None)

D. Reference

1. Kjeldahl, J.; Neue Methode zur Bestimmung der Stickstoffs in Organischen Korpern, Z. Anal. Chem., Vol. 22, 1883, pp. 366 - 378.

    A description of the author's original technique for measuring organic nitrogen.

3. 3. 22 <u>Bilirubin in Blood serum or plasma</u>

    A. Present methodology

        1. Icterus index[9]

            With the exception of carotene, bilirubin is the only normally occurring substance found in blood serum which has a strong absorbance at 455 mµ. The commonly used method is to compare the absorbance of a sample of blood serum in a colorimeter or spectrophotometer with that of a series of artificially prepared standards[1, 2, 3, 7, 15]

        2. Van den Bergh Method

            This test is based on the spectrophotometric determination of the diazo derivative which results from treatment of bilirubin with diazotized sulfanilic acid.[10, 11, 14]

        3. Rutkowski-deBaare Method[12]

            This is an ultramicro modification of the van den Bergh method. It requires the use of only 20 microliters of blood serum with no apparent sacrifice of accuracy.

    B. Applicability of present methodology to space flight conditions

        1. Merit table

Bilirubin

Merit Parameters	Icterus Index	van den Bergh	Rutkowski
1. Sensitivity	< 0. 1 mg%	< 0. 1 mg%	< 0. 1 mg%
2. Sample size	0. 25 ml.	0. 2 ml.	0. 02 ml.
3. Time required	3 min	5 min	5 min
4. Reproducibility	5	8	8
5. Suitability for null gravity use	-	-	-
6. Overall safety	10	5	5
7. Nontoxic reagents	10	3	3
8. Specificity	6	9	9
9. Insensitive to environmental changes	10	10	10

Bilirubin - continued			
Merit Parameters	Icterus Index	van den Bergh	Rutkowski
10. Ease in training personnel	10	10	8
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	10	8	8
13. Common use of analytic equipment	10	10	10
14. Nondestructive of sample	10	0	0
15. Merit range	23-46	22-44	22-44
16. Mean figure of merit	34	33	33

2. Discussion

The proposed methods are based on established time-tested procedures, and normal values by these methods are well established.

a. Icterus index

Carotene, lipemia and hemolysis interfere with accuracy. The method does not allow for differentiation between "free" and conjugated bilirubin which would be useful in the differential diagnosis of biliary obstruction and hepatocellular or hemolytic jaundice. These disadvantages may be offset, however, by the simplicity of the test.

b. Van den Bergh test

This method is more specific than icterus index but slightly more involved. The reagents have been prepared in the form of a compressed tablet[13] which should be simple to handle in a space environment. The use of this method has been reported to give excellent results.[8] In order to determine indirect (conjugated) bilirubin, methyl alcohol or a similar organic solvent is generally used.

c. Rutkowski-deBaare method

Because of the small sample of blood serum required and

the apparent sensitivity and accuracy of this method, it is preferred over the standard van den Bergh method. It suffers the disadvantage of requiring four reagent solutions, one of which is 60 percent phosphoric acid.

C. Areas for research and development

The simplicity and time-honored usefulness in clinical medicine of the presently employed tests for bilirubin, suggest that for purposes of monitoring the health of astronauts, these methods may be adequate.

More refined methods based on the fluorescence of oxidation products of bilirubin might be a useful approach to the development of more specific, highly sensitive methods of analysis.[4,5,6]

Other porphyrins also yield fluorescent oxidation products which may interfere. It should be noted that these studies were reported before the art of fluorescence analysis was well developed. Careful examination of the fluorescence spectra of bilirubin and related porphyrins, using different wavelengths for excitation may prove fruitful.

D. References

1. Bilissis, P.K.; Speer, R.J.; A Stable "Bilirubin" Standard Clin. Chem., Vol. 9, 1963, p. 552.

    An aqueous solution of N-(1 naphthyl) ethylenediamine - di - H Cl is a stable standard for bilirubin determination.

2. Bolshev, I.N.; Apparatus for Determining Bilirubin in Small Amounts of Blood, Sovrem. Probl. Gematol. i Perelin. Krovi, Vol. 36, 1964, p. 183.

    Determined bilirubin by icterus index in 3 mm capillary tubes by visual comparison with artificial standards prepared from bromthymol blue. Claims the method to be accurate and applicable for studies on newborn infants.

3. Cantarow, A.C.; Trumper, M.; Hepatic Function in Clinical Biochemistry, W.B. Saunders Co., 4th edition, 1950, p. 425.

    A discussion of the various clinical chemical methods for

determining bilirubin, including interpretation of the results.

4. Dhere, C.; Roche, J.; Fluorescence of Pigments of the Urobilin Group and Determination of their Fluorescence Spectrum, Bull. Soc. Chim. Biol., Vol. 13, 1931, p. 987.

    Compares the fluorescence spectra of several bilirubin-related compounds.

5. Dhere, C .; The Red Fluorescence which Certain Bilirubin Derivatives Show in Ultraviolet Light, Compt. Rend. Soc. Biol., Vol. 103, pp. 371 - 374.

    Describes the fluorescence spectrum of the product resulting from treatment of bilirubin with ammonia and zinc acetate.

6. Dhere, C.; Spectroscopical Study of a Bilirubin Derivative with Red Fluorescence, Arch. Intern. Pharmacodynamic, Vol. 38, 1930, pp. 134 - 139.

    The fluorescence spectra of compounds prepared by action of zinc acetate, iodine and potassium iodide were examined.

7. Fog, J. Serum Bilirubin and the Yellow Color of Serum, Scand. J. Clin. Lab., Invest., Vol. 10, 1958, pp. 251 - 256.

    Obtained good correlation between icterus index and bilirubin concentration of normal serum.

8. Hart, C.; Plaut, D.; Evaluation of a Bilirubin Screening Test, Am. J. Clin. Pathol., Vol. 45, 1966, pp. 510 - 511.

    Compressed tablet preparation containing sulfanilic acid and sodium nitrite was found to be efficient, rapid and reliable for routine clinical bilirubin determination.

9. Henry, R. J.; Golub, O. J.; Borkman, S.; Segalove, M.; Am. J. Clin. Pathol., Vol. 23, 1953, p. 841.

    Describes a widely used procedure for doing icterus index of blood serum.

10. Martinek, R. G.; Improved Micromethods for Determination of Serum Bilirubin, Clin. Chim. Acta., Vol. 13, 1966, pp. 161 - 170.

    A simple, rapid and precise diazo method is described which

requires 0. 1 ml. of serum. Interferences were resolved by improved kinetics and stoichiometry. Reliability of the method was established by demonstrating the lack of effect of lipemia and hemolysis.

11. Rand, R. N.; Di Pasqua, A.; A New Method for the Determination of Bilirubin, Clin. Chim. Acta., Vol. 8, 1962, pp. 570 - 578.

   The use of a 2-4 dichloroaniline instead of sulfanilic acid provided a simple method of determination. May be used for direct and indirect reading bilirubin.

12. Rutkowski, R. B.; de Baare, L.; An Ultramicro Colorimetric Method for Determination of Total and Direct Serum Bilirubin, Clin. Chem., Vol. 12, 1966, pp. 432 - 437.

   Describes a method which requires only 20 μl of blood serum. An improved diazo coupling reagent is utilized and phosphoric acid is used to prevent turbidity in the reaction mixture. Coefficient of variation of 15 replicate determinations on a 5 mg% standard was 3. 4%.

13. Sherman, L.; Diagnostic Preparation for Determination of Serum Bilirubin, U.S. Patent 2, 737, 501, March 6, 1956.

   A stable composition in tablet form which contains sodium sulfanilate and sodium nitrite in a water soluable binder is described for the determination of serum bilirubin.

14. Van den Bergh, A. A. H.; Miller, P.; Direct and Indirect Diazo Reaction with Bilirubin, Biochem. Z., Vol. 77, 1916, pp. 90 - 103.

   The original description of the van den Bergh method.

15. With, T. K.; Spectral Absorption of Bilirubin Measurements in Pure Aqueous Solutions Containing Human Serum. Acta., Physiol. Scand., Vol. 10, 1945, pp. 172 - 180.

   In pure aqueous solutions, maximum absorbance is at 420 mμ. In solutions containing serum, maximum absorbance is at 455 mμ. Change is due to formation of bilirubin-serum complex.

3.3.23 <u>Creatine and creatinine</u>

A. Principles of present methodology

Creatine can be measured in urine and protein-free blood solutions by the Voges and Proskauer reaction.[6] A fluorometric procedure has also been described which involves the reaction of creatine and ninhydrin in an alkaline solution.[4] The latter method is reported not to be very specific.[7]

For the present application creatine and creatinine are both to be measured and it would appear most economical and convenient to utilize a single technique for both. This can be done by employing a method for measuring creatinine. Creatine can be readily and essentially quantitatively converted to creatinine. Thus in one aliquot of urine, creatinine is measured and in another portion creatine is dehydrated to creatinine and creatinine is re-measured. The difference in the two analyses represents the creatinine formed from creatine.

There are three methods (colorimetric) which have been used to measure creatinine. One involves the use of 3,5-dinitrobenzoate in an alkaline solution.[2] This technique has not enjoyed great popularity because of its lack of specificity and the limited concentration range where it may be used. The procedure of Van Pilsum, <u>et al</u>.,[9] is probably the best method for measuring creatinine, but the large number of manipulations involved and the required use of an unstable reagent preclude its use for the present application.

The technique apparently most suitable for the present situation is one employing the Jaffe reaction.[8] This consists of the reaction of picrate and creatinine in an alkaline medium. A protein-free blood filtrate or unmanipulated urine is used for the reaction.

The application of this technique for the measurement of urine creatine and creatinine has received considerable study.[1] It is not so specific as some other methods,[5] but it is generally considered to be valid enough for most applications. A very desirable aspect of this

is the simplicity and the reagent stability. This is the only method
the compiler can recommend for the current application.

B. Suitability of present methodology to space flight conditions

   1. Merit table

An evaluation of the selected method for measuring creatine
and creatinine is shown tabulated below. Dimensionless numbers
are merit values.

Creatine and Creatinine

Merit Parameters	Serum Creatinine	Urine Creatinine	Urine Creatine
1. Sensitivity	Fair	Very Good	Fair
2. Sample size	100 µl	30 µl	30 µl
3. Time required	20 min	10 min	45 min
4. Reproducibility	7	9	6
5. Suitability for null gravity use	-	-	-
6. Overall safety	5	5	4
7. Nontoxic reagents	2	2	2
8. Noncaustic reagents	1	1	1
9. Specificity	5	8	7
10. Applicability	3	9	6
11. Reagent volatility	10	10	10
12. Insensitive to environmental changes	1	1	1
13. Analyst training	5	10	7
14. Manipulations	5	10	3
15. Common use of analytic equipment	10	10	10
16. Merit range	10-20	19-38	9-19
17. Mean figure of merit	15	29	14

   2. Discussion

The method which appears most promising for near-future use
is one utilizing the Jaffe reaction[6] for creatinine and creatine.

Creatine and creatinine are in equilibrium in solution. The

equilibrium constant for the reaction is known to be a function of pH and temperature.[3] It seems reasonable that conditions could be found which would favor the accumulation of creatine. If the creatine formed could be "trapped" by the formation of a derivative or complex, it would then be possible to effect a quantitative conversion of creatinine to creatine and then the Voges and Proskauer[6] reaction or perhaps even a fluorometric technique[4] could be employed for measuring creatine and creatinine. The advantage of this approach is that the Voges and Proskauer reaction is probably much more specific for creatine that the Jaffe reaction[8] is for creatinine. The "trapping" of the creatine might be accomplished by the use of a cationic exchange resin. The amidine group is a strong base and should form a stable complex with acidic resins.

C. Areas for research and development

The mono-substituted amidine group of creatine and the disubstituted amidine of creatinine provide quite reactive sites for the formations of derivatives with characteristic optical properties. Perhaps techniques suggested for forming derivatives of the amino acids could be utilized.

D. References

1. Biggs, H.G.; Cooper, J.M.; Modified Folin Methods for the Measurement of Urinary Creatine and Creatinine, Clin. Chem., Vol. 7, 1961, pp. 655 - 664.

   Presents a simplified method for converting creatine to creatinine and describes a method of using the Jaffe reaction to measure both.

2. Bollinger, A.; The Colorimetric Determination of Creatinine in Urine and Blood with 3,5-Dinitrobenzoic Acid, Med. J. Australia, Vol. 2, 1936, pp. 818 - 821.

   Describes a procedure for the assay of creatinine using 3,5-dinitrobenzoate and 1 N NaOH. Best range is 2 - 15 mg%.

3. Brinkerink, P.C.; Determination of Creatine in Urine, Clin. Chim.

Acta, Vol. 6, 1961, pp. 532 - 537.

Studied the equilibrium of creatine and creatinine under the various conditions, particularly as related to measurement of creatinine.

4. Conn, R. B.; Fluorometric Determination of Creatine, Clin. Chem., Vol. 6, 1960, pp. 537 - 548.

Assay of creatine by fluorescence following reaction with ninhydrin in an alkaline media.

5. Cooper, J. M.; Biggs, H. G.; An Evaluation of Four Methods of Measuring Urinary Creatinine, Clin. Chem., Vol. 7, 1961, pp. 665 - 673.

Compared the methods of Folin, Hare, Van Pilsum and Sullivan and Irreverre. Found the latter method to yield erroneous results whereas the others provided essentially the same results.

6. Ennor, A. H.; Stocken, L. A.; The Application of the Diacetyl Reaction to the Estimation of Creatine in Urine, Biochem. J., Vol. 55, 1953, pp. 310 - 314.

Applied the Voges and Proskauer reaction for the measurement of creatine in urine. Avoided all references to Voges and Proskauer.

7. Henry, R. J.; Clinical Chemistry, Principles and Techniques, Hoeber Medical Division, Harper and Row, New York, 1964, p. 291.

A book devoted entirely to the principles and techniques of chemistry. The author generally discusses several methods in some detail and then selects one or two and describes them in great detail. Provides values of accuracy, precision and normal values for selected procedures.

8. Jaffe, M.; Uber den Niederschlag welchen Pikrinsaure in normalen Harn erzeugt, und uber eine neue Reaction des Kreatinim, Z. Physiol. Chem., Vol. 10, 1886, p. 391.

Described the chromogenic reaction of picric acid and creatinine in an alkaline medium.

9. Van Pilsum, J. F., et al.; Determination of Creatine, Creatinine, Arginine, Guanidoacetic Acid, Guanidine and Methyl Guanidine in

Biological Fluids, J. Biol. Chem., Vol. 222, 1956, pp. 225 - 236.

Measured creatinine by degradation to methyl-guanidine with o-nitrobenzaldehyde and Sakaguchi reaction. Creatine was determined by prior dehydration to creatinine.

3.3.24 Lactic acid in serum

A. Present methodology with modifications
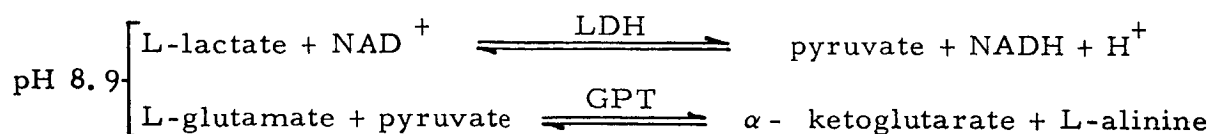
1. Method 1

Compared to many other methods for measuring lactic acid, the fluorometric method of Loomis[1] has a distinct advantage since no protein precipitating step is required. The native fluorescence of different sera is not significantly different, and only one serum blank is needed for a series of tests. The reaction is as follows:

$$\text{L-lactate} + \text{NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$$
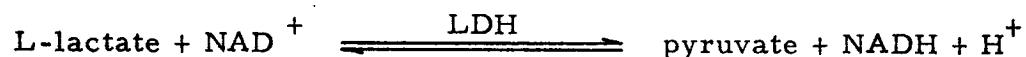
The reaction is carried to completion by conducting it at pH 10.5 and by using semicarbazide to trap the pyruvate. The method has good precision and adequate sensitivity. However, the reaction rate is slow (requires a 90 minute incubation period at room temperature) and for this reason modifications are recommended (see Method 3.)

2. Method 2

A method for measuring lactic acid recently developed by Noll[2] requires only a 20 minute incubation period. This rapid reaction rate was achieved by coupling the lactate dehydrogenase (LDH) and glutamate-pyruvate transaminase (GPT) reactions whereby pyruvate is converted to L-alanine by reaction with excess glutamate. Pyruvate is therefore rapidly removed from the equilibrium of the lactate dehydrogenase reaction:

$$\text{pH } 8.9 \begin{cases} \text{L-lactate} + \text{NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+ \\ \text{L-glutamate} + \text{pyruvate} \xrightleftharpoons{\text{GPT}} \alpha\text{-ketoglutarate} + \text{L-alinine} \end{cases}$$

In addition to being required in the reaction, glutamate also serves as the buffer. As in the previous method, the product that is measured is NADH, but in this case it is measured spectrophotometrically at 366 mμ. The method has good precision and adequate sensitivity. Its primary disadvantage is that the analysis is per-

formed on a protein free filtrate. For this reason modifications are recommended (See Method 3.)

3. Method 3

   Proposed modified Loomis-Noll method for measuring lactate. Loomis[1] has demonstrated that it is not necessary to deproteinize serum before fluorometric analysis of its lactate content. Noll[2] has demonstrated that the time required for the reaction to go essentially to completion can be significantly shortened. Thus it appears that the good points of the two methods can be combined to yield a method that is better than either. It also appears that the required reagents can be premixed in solution, apportioned into the tubes, and lyophilized before being carried into space. A general outline of the proposed modified method is as follows:

   a.   A solution (pH 8.9 glutamate buffer) containing the proper amounts of NAD^+, LDH, L-glutamate and glutamate pyruvate transaminase would be prepared on earth and apportioned into tubes that would ultimately be used as cuvettes for the fluorometer.

   b.   The contents of the cuvettes would immediately be lyophilized, sealed, and stored under refrigeration until used in space.

   c.   In space, the lyophilized material could be reconstituted by adding the proper amount of water at the time the experiment is to be conducted.

   d.   Serum would then be added (no protein precipitation required) to the reaction mixture and incubated at room temperature for 20 minutes.

   e.   The NADH produced during incubation would be measured fluorometrically.

   f.   A single serum blank and appropriate standards would also

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be required. The amount of serum required for this modified procedure should be 50 µl or less.

The method should have good precision, adequate sensitivity, and only a minimum of time and training will be required. Consequently, this proposed procedure is recommended for measuring lactate during space flight.

B. Suitability of present methodology to space flight conditions

　　1. Merit table

An evaluation of the methods for lactic acid is presented below in tabular form. Dimensionless numbers are merit values; the greater the value, the more desirable.

Lactic Acid

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	Good
2. Sample size	100 µl	200 µl	50 µl
3. Time required	100 min	20 min	20 min
4. Reproducibility	9($\pm$ 2%)	9($\pm$ 2%)	9($\pm$ 2%)
5. Suitability for null gravity use	8	7	9
6. Overall safety	10	10	10
7. Nontoxic reagents	10	10	10
8. Noncaustic reagents	10	10	10
9. Specificity	10	10	10
10. Insensitive to environmental changes	9	9	9
11. Ease in training personnel	7	6	9
12. Degree of separation required (10=none)	9	7	9
13. Minimal handling by analyst	9	7	9
14. Common use of analytic equipment	10	10	10
15. Nondestructive of sample	0	0	0
16. Figure of merit	73	66	79

2. Discussion

In the modified method (Method 3) recommended for measuring blood lactate, the reaction is carried out under essentially the same conditions as recommended by Loomis.[1] Quantitation is accomplished by fluorometric measurement of the native fluorescence of the NADH produced as recommended by Loomis.

In theory the modifications should result in an improved method, but this can be verified only by laboratory experiments. Since the modifications are relatively minor, only a short period of laboratory experimentation should be required to perfect the method for space use.

In the method of Loomis large volumes are used (0.1 ml of serum diluted to a final volume of approximately 16 ml before measurement in the fluorometer). By reducing the volumes used, one should need only 25 - 50 µl of serum for each determination.

Equipment

Method 1: Fluorometer

Method 2: Spectrophotometer

Method 3: Fluorometer
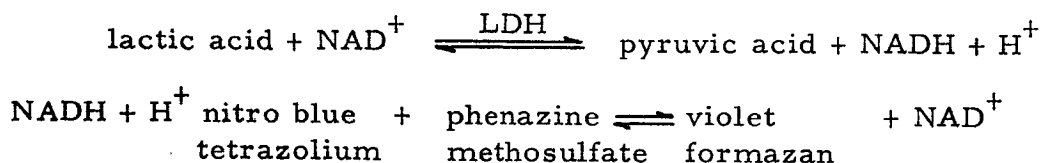
Method 4: Densitometer (See part C)

A centrifuge will be needed to separate the serum from the blood clot.

C. Areas for research and development (Method 4)

Another possible approach to the problem of measuring lactate is one similar to that which is commonly used to measure LDH activity. This approach is briefly outlined below:

1. Impregnate filter paper with the following reagents:

   a. Phosphate buffer (pH 7.4)

   b. Sodium cyanide

   c. Nitro blue tetrazolium

    d. Phenazine methosulfate

    e. NAD^+

    f. Lactic dehydrogenase

2.   The dried substrate should be stable in filter paper if properly stored.

3.   When serum is applied to filter paper impregnated with substrate the following reactions should occur:

$$\text{lactic acid} + NAD^+ \xrightleftharpoons{\text{LDH}} \text{pyruvic acid} + NADH + H^+$$

$$NADH + H^+ \underset{\text{tetrazolium}}{\text{nitro blue}} + \underset{\text{methosulfate}}{\text{phenazine}} \rightleftharpoons \underset{\text{formazan}}{\text{violet}} + NAD^+$$

With a densitometer one should be able to make a semi-quantitative estimate of the amount of lactate present by comparing the intensity of the formazan spot produced by serum with that produced by a standard lactate solution.

D. References

1. Loomis, M. E.; An Enzymatic Fluorometric Method for the Determination of Lactic Acid in Serum, J. Lab. Clin. Med., Vol. 57, 1961, pp. 966 - 969.

    This method is a simplified adaptation to fluorometry of an existing spectrophotometric method for the determination of serum lactic acid. The analysis is performed directly on serum without protein precipitation.

2. Noll, F.; Methode zur quantitativen Bestimmung von L (+) -Lactate mittels Lactat-Dehydrogenase und Glutamat-Pyruvat-Transaminase, Biochem. Z., Vol. 346, 1966, pp. 41 - 49.
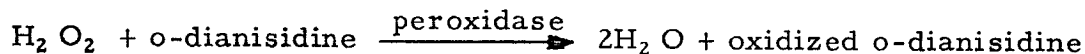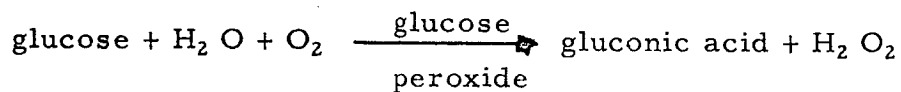
    The author achieved a very rapid reaction rate by coupling the lactate dehydrogenase and glutamate-pyruvate transaminase reactions whereby pyruvate is converted to L-alanine by reaction with excess glutamate. Pyruvate is therefore rapidly removed from the equilibrium of the lactate dehydrogenase reaction. The analysis is performed on a protein free filtrate. One of the products of the reaction (NADH) is measured spectrophotometrically at 366 mμ.

3. 3. 25 <u>Blood sugar (glucose)</u>

A. Present methodology and recommendations

1. Method 1

A modification of the method of Cawley <u>et al.</u>,[1] is recommended for measuring blood glucose. The reactions involved in this method are outlined below:

$$glucose + H_2O + O_2 \xrightarrow{\text{glucose peroxide}} gluconic\ acid + H_2O_2$$

$$H_2O_2 + o\text{-dianisidine} \xrightarrow{\text{peroxidase}} 2H_2O + oxidized\ o\text{-dianisidine}$$

In this method serum or plasma may be used directly <u>without a protein precipitating step.</u> The product of the above reactions (oxidized o-dianisidine) is stable and is measured at 400 mμ. The method requires only 25 μl of serum or plasma and values obtained compare favorably with those obtained by the classical methods for measuring glucose.[1]

Hemolyzed and icteric serum or plasma cannot be used in this procedure. Other interferring substances commonly found in serum and plasma do not cause significant error under the conditions of the procedure (the serum is diluted with excess enzyme.)

In order to render the method more adaptable to space use, the following modifications are recommended:

1. A solution containing the proper amounts of the required enzymes and reagents should be prepared on earth, apportioned into tubes that will ultimately be used as cuvettes, frozen, lyophilized, and stored under refrigeration until used in space. The premixed lyophilized reagents should be stable since they are essentially the same as those used in the "dip stick" method for glucose. In "dip sticks" the premixed reagents have been found to be stable if the proper amount of gelatin is included in the reagent mixture.

2. The lyophilized material can be reconstituted in space by adding the proper amount of water at the time a glucose determination is to be made. Serum can now be added and incubation commenced.

3. It is recommended that the incubation be carried out at 37° C for 30 minutes rather than under the conditions used by Cawley et al. The longer incubation time at a higher temperature permits the reaction to go essentially to completion and should eliminate the necessity of stopping the reaction with a drop of HCl provided measurements are made within a short time after incubation is completed.

The method has good precision, adequate sensitivity and only a minimum of training is required.

2. Method 2

Thompson[3] has published a colorimetric glucose oxidase method which is approximately four times as sensitive as similar glucose oxidase methods (those requiring a protein free filtrate). In this procedure the peroxidase reaction is eliminated. $H_2 O_2$ produced by the glucose oxidase reaction reacts with a molybdate catalyst and with I^- to yield I_2, which in turn reacts with o-tolidine to yield a chromogen that is measured at 620 mμ.

When considered for space use the method has the following disadvantages:

a. A protein precipitating step is required.

b. The stability of the reagents when premixed and lyophilized is unknown. It may be possible to eliminate the protein precipitating step just as Cawley, et al.,[1] have done, and the premixed, lyophilized reagents may be stable. These uncertainties can be resolved only by experimentation. If the method can be modified to eliminate the above disadvantages, it should be superior to Method 1.

3. Method 3

Despite the limitations of the "dip stick" method[2] for measuring glucose, simplicity makes it attractive. Therefore the possibility of improving its accuracy should be investigated.

Increasing the enzyme concentration of the "dip stick" should help to minimize the effect of interferring substances present in blood. A search for better chromogenic oxygen acceptors is also needed.

It would probably be worthwhile to test the accuracy of "dip sticks" on earth on each individual who will later need glucose measurements in space. The wide variation in glucose values of certain individuals as measured by "dip sticks" may reflect biological differences that one would not find in a healthy astronaut population. In its present state, however, the method appears to have neither adequate precision nor adequate sensitivity for quantitative glucose measurements.

B. Suitability of present methods to space flight conditions

1. Merit table

An evaluation of the methods for glucose is presented on the following page. Dimensionless numbers are merit values; the greater the value, the more desirable.

2. Discussion

The recommended method and modifications thereof should be adequately tested in the laboratory before being used in space. Since the modifications are minor, this should require only a short period of time. The following major instruments would be needed:

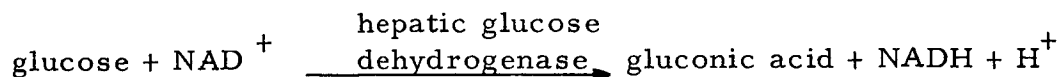| Method 1 | Spectrophotometer |
Method 2	Spectrophotometer
Method 3	None
Method 4	Fluorometer

A centrifuge will also be needed to separate the serum from the blood clot.

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Glucose

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	Good
2. Sample size	25 µl	200 µl	20 µl
3. Time required	35 min	60 min	1 min
4. Reproducibility	8($\pm$ 3%)	8($\pm$ 3%)	2($\pm$ 20%)
5. Suitability for null gravity use	8	7	9
6. Overall safety	10	10	10
7. Nontoxic, noncaustic reagents	10	10	10
8. Specificity	10	10	10
9. Insensitive to environmental changes	9	9	9
10. Ease in training personnel	9	7	9
11. Degree of separation required (10=none)	9	7	10
12. Minimal handling by analyst	9	7	9
13. Common use of analytic equipment	10	10	10
14. Nondestructive of sample	0	0	0
15. Figure of merit	73	65	17

C. Areas for research and development

Method 4 (to be developed)

In theory it should be possible to measure glucose by the following reaction:

$$\text{glucose} + \text{NAD}^+ \xrightarrow{\text{hepatic glucose dehydrogenase}} \text{gluconic acid} + \text{NADH} + \text{H}^+$$

The NADH produced could be measured fluorometrically and would be directly related to the amount of glucose present in the serum. Such a fluorometric procedure should be much more sensitive than the method recommended for measuring glucose. However, in order to develop such a method one would first have to purify hepatic glucose dehydrogenase since it is not commercially available in a highly purified form. Consequently a great deal of time and effort would probably be required for development of the method.

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D. References

1. Cawley, L.P.; Spear, F.E.; Kendall, R.; "Ultramicro Chemical Analysis of Blood Glucose with Glucose Oxidase," Am. J. Clin. Path., Vol. 32, 1959, pp. 195 - 200.

   A coupled enzyme system of glucose oxidase and peroxidase is used in combination with a chromogenic oxygen acceptor, and the analysis performed directly on 25 μl of serum or plasma without protein precipitation. The product of the reaction is measured at 400 mμ. The effect of common interferring substances is essentially eliminated by incubating the serum in a large excess of enzyme.

2. Joyner, R.E.; Reagent-Strip Method of Blood Glucose Determination, J. Occupational Med., Vol. 7, 1965, pp. 512 - 515.

   In most patients the Dextrostix[*] value for glucose was found to be within $\pm$ 10 mg% of the glucose value obtained by classical methods. However 20 of 70 patients were outside the $\pm$ mg% range with five values being in error by more than 20 mg%.

3. Thompson, R.H.; Colorimetric Glucose Oxidase Method for Blood Glucose, Clin. Chim. Acta., Vol. 13, 1966, pp. 133 - 135.

   In this procedure the peroxidase reaction is eliminated. $H_2 O_2$ produced by the glucose oxidase reaction reacts with a molybdate catalyst and with I^- to yield I_2 which in turn reacts with o-tolidine to yield a chromogen that is measured at 620 mμ.

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[*] Registered trademark of Ames Co., Inc., Elkhart, Indiana

3.3.26 Bicarbonate in serum

A. Principles of present methodology

There are several volumetric, manometric, and colorimetric procedures for the measurement of bicarbonate. The preferred method however, would be the non-destructive electrometric assay. The usual technique is to use pH sensitive electrodes in a very dilute carbonic acid bicarbonate buffer solution.[1] This solution is separated from the specimen to be analyzed by a membrane permeable to carbon dioxide. The carbon dioxide in the specimen and the buffer will equilibrate and the resulting pH (carbon dioxide concentration) of the specimen. A concomitant direct measurement of the specimen pH allows a simple calculation of the bicarbonate concentration in that they are related as follows:

$$pH = pK + \log \frac{[HCO_3]}{[H_2 CO_3]} \qquad (1)$$

where pH for serum = 6.1 and brackets indicate concentration. Since

$$[H_2 CO_3] = pCO_2 \times 0.03,$$

equation (1) can also be written as

$$pH = pK + \log \frac{[HCO_3]}{[pCO_2 \times 0.03]} \qquad (2)$$

There are other methods utilizing electrometric measurements, but the one described appears most expedient. The equipment is commercially available and quite reliable.

B. Suitability of present method to space flight conditions

Bicarbonate in Serum

Merit Parameters	Electrometric Assay
1. Sensitivity	Good
2. Sample volume	50 μl
3. Time required	5 min
4. Reproducibility	10

| Bicarbonate in Serum - continued	
Merit Parameters	Electrometric Assay
5. Suitability for null gravity use	-
6. Overall safety	10
7. Nontoxic reagents	9
8. Noncaustic reagents	8
9. Specificity	9
10. Applicability	10
11. Reagent volatility	9
12. Insensitive to environmental changes	9
13. Analyst training	8
14. Manipulation	9
15. Common use of analytic equipment	2
16. Merit range	44-87
17. Figure of merit	65

C. Areas for research and development

Future efforts should probably be directed toward further development of the electrometric technique. Instrument stability and maintenance are areas which undoubtedly need the greatest attention.

One of the problems encountered with the electrometric measurement of bicarbonate is the slow response time. The time required for a valid measurement is apparently due to the fact that the reactions

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad (3)$$
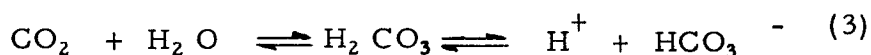
are slow to reach equilibrium.

The use of enzymes in conjunction with electrometric assays has been described[2] and in one application carbonic anhydrase has been used with a pCO_2 electrode system.[3] The technique utilizes carbonic anhydrase and bicarbonate incorporated in methylated cellulose. The enzyme catalyzes the hydration-dehydration of carbon dioxide,

$$CO_2 + H_2O \xrightleftharpoons[\text{anhydrase}]{\text{carbonic}} H_2CO_3, \qquad (4)$$
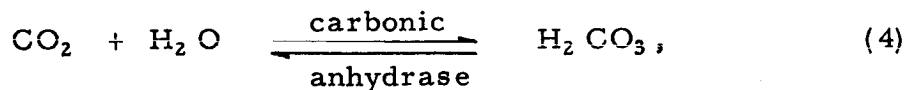
so that the equilibrium for reaction (3) is reached more quickly.

A difficulty with the use of carbonic anhydrase is instability of the enzymes. A low pCO_2, which results in an elevated pH in the buffer-electrolyte solution, inactivates the enzyme.[3] Two possible ways of avoiding this problem would be to use an electrolyte with a greater buffer capacity so that the pH changes would be less, or to employ a more pH-resistant carbonic anhydrase. The enzymes used have been obtained from erythrocytes. Other sources, perhaps parsley,[3] might yield a more resistant enzyme.

The use of carbonic anhydrase in this system requires a considerable investigation before it can be recommended.

D. References

1. Jensen, O. J.; Direct pO_2 and pCO_2 Measurement. Labatorium No. 3, 1963, pp. 2 - 7.

   Discusses the principles of the direct measurement of pO_2 and pCO_2.

2. Clark, L. C., Jr.; Lyons, Champ; Electrode Systems for Continuous Monitoring in Cardiovascular Surgery, N. Y. Acad. Sci., Vol. 102, 1962, pp. 29 - 45.

   Authors describe the use of certain enzymes in conjunction with electrometric techniques.

3. Clark, L. C., Jr. Dept. of Surgery, Univ. of Ala. Medical Center, Birmingham, Alabama; Personal communication, 1967.

3.3.27 <u>Chloride in serum, plasma, urine, feces, and sweat</u> .

    A. Principles of present methods

        There are several colorimetric procedures for estimating chloride. Chlorides can also be assayed amperometrically. However, for the present application it appears that the method of choice would be electrometric. This is a non-destructive physical-chemical technique which presumably could be used for all the specimens. A recent publication describes the automated potentiometric determination of four inorganic ions including chloride.[1] The author did not experience "protein fouling" with his electrodes which has been a previous problem with similar systems. Electrometric methods measure activity instead of concentration, but the difference should not be great and the results should not be difficult to interpret.

    B. Suitability of present method to space flight conditions

<div align="center">Chloride</div>

Merit Parameters	Electrometric Chloride Assay
1. Sensitivity	–
2. Sample volume	200 µl *
3. Time required	60 sec
4. Reproducibility	10
5. Suitability for null gravity use	–
6. Overall safety	10
7. Nontoxic reagents	9
8. Noncaustic reagents	10
9. Specificity	10
10. Applicability	10
11. Reagent volatility	10
12. Insensitive to environmental changes	9
13. Analyst training	9
14. Manipulation	8

* Estimated from Dahms' data.

Chloride - continued

Merit Parameters	Electrometric Chloride Assay
15. Common use of analytic equipment	2
16. Merit range	45-90
17. Mean figure of merit	68

C. Areas for research and development

Future efforts should probably be directed toward further development of electrometric devices. This approach seems well-suited for the intended application: non-destructive, fast, accurate and requires small amounts of material. The long term stability of the electrodes and maintenance problems need evaluation.

D. Reference

1. Dahms, H.; Automated Potentiometric Determination of Inorganic Blood Constituents (Na^+, K^+, H^+Cl^-), Clin. Chem., Vol. 13, 1967, pp. 437 - 450.

Describes an automated system for electrometric measurements. Fast and accurate data processing interfacing also.

3.3.28 <u>Phosphate and pyrophosphate in serum and urine</u>

A. Principles of present methodology

There are numerous techniques which have been employed to assay inorganic phosphate in biological materials. Most of these which are sensitive enough for the micro-sampling required for the current application are colorimetric.

The literature search revealed few procedures for the estimation of inorganic pyrophosphate in the same sample with the same reagents.[1] The differentiation is made on the basis of time. When a sulfhydryl compound (cysteine) is incorporated as a reagent, the color due to orthophosphate is produced maximally in 5 - 7 minutes, whereas the color due to pyrosphosphate is not fully developed for 90 minutes. Thus the same specimen, under the same conditions can be used to colorimetrically measure orthophosphate at 7 minutes and the difference in color intensity (absorbance) at 90 - 7 minutes can be used as a measure of pyrophosphate.

B. Suitability of present method to space flight conditions

Phosphate and Pyrophosphate

Merit Parameters	Inorganic Phosphate	Inorganic Pyrophosphate
1. Sensitivity	Fair	-
2. Sample volume	100 μl*	87 μg **
3. Time required	30 min	110 min
4. Reproducibility	8	-
5. Suitability for null gravity use	-	-
6. Overall safety	2	2
7. Nontoxic reagents	1	1
8. Noncaustic reagents	1	1
9. Specificity	9	-
10. Applicability	2	2
11. Reagent volatility	3	3

* Volume of serum with concentration of 3.5 mg% inorganic phosphate which would would provide an absorbance of 0.09.[1]

Phosphate and Pyrophosphate - continued

Merit Parameters		Inorganic Phosphate	Inorganic Pyrophosphate
12.	Sensitivity to environmental changes	7	7
13.	Analyst training	5	5
14.	Manipulation	5	6
15.	Common use of analytic equipment	10	10
16.	Merit range	9-19	0-24
17.	Mean figure of merit	14	8

The mechanism of the color reaction for pyrophosphate is apparently not known, but a sulfhydryl compound is required and pyrophosphate is not hydrolyzed to orthophosphate.

Difficulty with this procedure will arise if the amount of pyrophosphate is small as compared with the amount of phosphate because the absorbance of the latter will tend to obscure the absorbance of the former. This in turn will yield little accuracy and precision for the assay of pyrophosphate.

2. Discussion

The estimation of urine pyrophosphate would require only a colorimeter, unless the urine contains protein or certain other abnormal constituents.

C. Areas for research and development

The procedure described above for phosphate and pyrophosphate analyses is not very desirable because of the reagents and the probable lack of sensitivity for pyrophosphate.

Neutron activation and analysis of the products would appear to be an area worthy of exploration. However, this type of analysis must be preceded by a separation of the two compounds. Ion-exchange[3] and differential precipitation[2] techniques have been used for separation.

** Amount of inorganic pyrophosphate required to yield an absorbance of 0.050.[1]

It should be simple to determine inorganic pyrophosphate directly by employing the enzyme pyrophosphatase and measuring phosphate before and after enzymic digestion.

Should colorimetric procedures evolve as more applicable, separation of the phosphorus compounds prior to analyses should provide greater sensitivity and probably greater accuracy and precision, particularly for pyrophosphate.

D. References

1. Flynn, R. M.; Jones, M. F.; Lipmann, F.; The Colorimetric Determination of Inorganic Pyrophosphate. J. Biol. Chem., Vol. 211, 1954, pp. 791 - 796.

   A procedure is described in which inorganic phosphate and inorganic pyrophosphate are measured sequencially. Differentiation is based on time; pyrophosphate reacts slower in presence of sulfhydryl.

2. Hoffmann, E.; Saraez, A.; Use of Chloranilic Acid Salts in Microanalysis, Z. Anal. Chem., Vol. 190, 1962, pp. 326 - 329.

   Chloranilic acid can be used to precipitate pyrophosphate, leaving orthophosphate in solution. Orthophosphate can be measured in supernatant solution and pyrophosphate can be quantitated on basis of absorbance of chloranilic acid released on acidification and solubilization of precipitated chloranilopyrophosphate.

3. Lindenbaum, S.; Peters, T. V.; Rieman, W.; Analysis of Mixtures of Condensed Phosphates by Ion-Exchange Chromatography, Anal. Chim. Acta., Vol. 11, 1954, pp. 530 - 537.

   Presents a technique for separating several polyphosphates by ion-exchange. Procedure requires 5 hours.

3.3.29 Manganese in whole blood or serum

A. Principles of present methods

##### 1. Method[1] - Papavasiliou and Cotzias[3]

Upon neutron bombardment, the stable isotope of manganese, Mn^{55}, becomes Mn^{56}, which decays by emission of beta and gamma rays. If activation conditions are constant the Mn^{56} produced is proportional to the parent element Mn^{55}. The Mn^{56} must be chemically separated from the mixture of radioisotopes (e.g. K, Na, Cl, etc.) which exert a masking effect. Separation is facilitated by the addition of carrier.

Procedure: The samples of blood or serum are dried at 85° C in polyethylene tubes. The tubes are sealed and introduced in the reactor for activation (2 hours). The contents of the tubes are washed in glass containers with hot HNO_3. Carrier is added to $(MnSO_4)$ and the solutions are digested and oxidized to MnO_4^-. The MnO_4^- is precipitated with tetraphenylarsonium chloride. The precipitate is filtered, washed and placed on planchets for assay of the radioactivity with a gamma-ray spectrometer.

The method has good reproducibility and specificity. The levels of manganese in blood and serum by this method are extremely low. Cotzias[1] reported mean values of manganese for serum and whole blood of 0.587 μg/L and 8.44 μg/L, respectively.

2. Method 2 - Fernandez, et al.[2]

Manganese ions in the presence of sodium periodate catalyze the oxidation of Malachite Green (MG) to colorless products. The disappearance of MG is measured at 620 mμ in a spectrophotometer. The reaction may be illustrated as follows:

$$Mn^{++} \xrightarrow{\quad NaIO_4 \quad} Mn^{oxidized}$$

$$Malachite\ green \xrightarrow{\quad Mn^{ox} \quad} colorless\ products$$
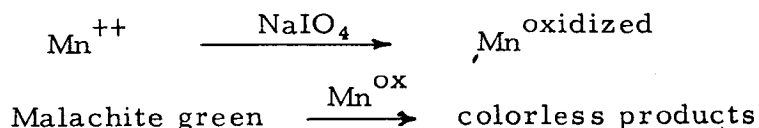
The reaction is of first order and therefore requires a kinetic

analysis. Absorbance measurements are taken at various time intervals and the logarithms of the absorbances are plotted against time. The manganese concentration is calculated from the slope of the line by comparison to the slope obtained from a known quantity of manganese:

$$m\mu g\ Mn = \frac{Kx - b}{Ks - b} \times S$$

Where:

> $m\mu g$ = millimicrograms
>
> Kx = slope of the sample
>
> Ks = slope of the standard
>
> S = $m\mu g$ of Mn in the standard
>
> b = slope of the blank

Procedure: Serum is dried at 116° C and then ashed in a muffle furnace at 540° C, until no carbon particles are visible.

The residue is dissolved in HCl and the pH of the acid solution is adjusted to 3.6 with a buffer. Malachite green and $NaIO_4$ are added and the samples are incubated at 26°. Spectrophotometric readings are taken every hour for five hours. The readings are commenced three (3) hours after the addition of $NaIO_4$. Blanks and standards are run along with the samples. The normals of values values for this method are similar to those reported by Cotzias, et al.[1] The normal range was .360 - .900 $\mu g/L$. The recovery of known amounts of Mn added to serum was approximately 76%.

B. Applicability of present methods to space flight conditions

    1. Merit table

Manganese

Merit Parameters	Method 1	Method 2
1. Sensitivity	Good	Good
2. Sample size	.1 ml	.2 ml
3. Time required	–	12 hours

Manganese - continued

Merit Parameters	Method 1	Method 2
4. Reproducibility	8	-
5. Suitability for null gravity use	2	2
6. Overall safety	0	0
7. Nontoxic reagents	0	6
8. Noncaustic reagents	0	5
9. Specificity	8	8
10. Insensitive to environmental changes	2	2
11. Ease in training personnel	1	1
12. Degree of separation required (10=none)	1	8
13. Minimal handling by analyst	0	1
14. Common use of analytic equipment	0	2
15. Nondestructive of sample	0	0
16. Merit range	-	0-14
17. Mean figure of merit	4	7

2. Discussion

Equipment

Method 1: Heater, reactor for neutrons, gamma-ray spectrometer, pulse height analyzer, oven, centrifuge, amplifier and power supply.

It may also be worthwhile to investigate the effects of serum manganese on enzymes requiring manganese, e.g., isocitric dehydrogenase.

Method 2: Centrifuge, spectrophotometer, muffle furnace, oven. Neither method can be recommended.

C. Areas for research and development

The isolation of Mn from serum must be achieved without acid digestion or ashing. Following its isolation the catalytic properties of the element can be applied for its measurement.

D. References

1. Cotzias, G.C.; Miller, S.T.; Edwards, J.; Neutron Activation Analysis: The Stability of Manganese Concentrations in Human Blood and Serum, J. Lab. Clin. Med., Vol. 67, 1966, pp. 836 - 849.

   Normal values of Mn in whole blood and serum are given. The serum level of Mn appears to be very constant.

2. Fernandez, A.A.; Sobel, C.; Jacobs, S.L.; Sensitive Method for the Determination of Submicrogram Quantities of Manganese and its Application to Human Serum, Anal. Chem., Vol. 35, 1963, pp. 1721 - 1724.

3. Papavasiliou, P.S.; Cotzias, G.C.; Neutron Activation Analysis: The Determination of Manganese, J. Biol. Chem., Vol. 236, 1962, pp. 2365 - 2369.

   Manganese was measured in blood, plasma and tissues by neutron activation analysis.

3.3.30 Sodium, potassium, magnesium, and calcium

A. Principles of present methodology

1. Flame photometry[3, 4]

Atomize a solution of the sample to be analyzed into a flame in order to effect ionization of the atoms of the metal present in the sample. Measure the quantity of light of characteristic wavelength which is emitted with a photomultiplier tube.

2. Atomic absorption[1]

The emission from a hollow cathode lamp which contains Na, K, Ca and Mg is passed through a flame into which a solution of the sample for analysis is aspirated. The quantity of light from the emission of the hollow cathode with characteristic wavelength of the element being determined which passes through the flame will be inversely related to the concentration of atoms of that element present in the flame.

3. Neutron activation analysis

The sample to be analyzed is placed in a high flux of thermal neutrons for a period of several minutes. Radionuclides of the elements present in the sample are produced as a result of reactions with neutrons. The energy of radiation emitted when these nuclides decay is characteristic of that element. By determining the decay rate of each radioactive element produced, one can calculate the concentration of the element present in the sample.

B. Applicability of present methodology to space flight conditions

1. Merit table

Merit Parameters	Flame Photometry	Atomic Absorption	Neutron Activation
1. Sensitivity	> 1 ppm	> 0.1 ppm	> 0.1 ppm
2. Sample size	< 0.1 ml	< 0.1 ml	< 0.1 ml
3. Time required	3 min	3 min	5 min

Merit Parameters	Sodium, potassium, magnesium, and calcium		
	Flame Photometry	Atomic Absorption	Neutron Activation
4. Reproducibility	10 (> 1%)	10 (> 1%)	10 (> 1%)
5. Suitability for null gravity use	5	5	10
6. Overall safety	5	5	5
7. Noncaustic, nontoxic reagents	7	7	5
8. Specificity	8	9	10
9. Insensitive to environmental changes	8	8	10
10. Ease in training personnel	8	8	10
11. Degree of separation required (10=none)	10	10	10
12. Minimal handling by analyst	9	9	10
13. Common use of analytic equipment	5	5	6
14. Nondestructive of sample	0	0	10
15. Figure of merit	42	43	64

2. Discussion

If the atmosphere in the spacecraft is 100% oxygen, a means must be devised for containing the burner used in flame photometry and atomic absorption. Also a means must be provided for eliminating the products of combustion. Lasers might be substituted for flames. Hollow cathode lamps have been described[2] into which a sample may be placed and ionized electrically in an inert atmosphere. This might serve as a better source of excitation than a flame. Shielding of personnel from the neutron source and of the radiation detector from cosmic radiation appear to be the major problems related to neutron activation analysis.

C. Areas for research and development

1. Investigation of the use of lasers in flame photometry and atomic absorption.

2. Specific ion electrodes.

This category of approach to sodium, potassium, calcium, and magnesium was placed in the promising area for research and

development because at the present time information regarding their clinical use is sketchy. This problem, however, should be easily solved. This area undoubtedly represents the most fruitful approach for research and development for clinical measurements under space flight conditions. Equipment required is relatively simple[5]. A well designed high impedance voltmeter could serve for multiple measurements. It is possible that variations of specific ion electrodes can be used for other methodologic approaches.

D. References

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   A book which reviews the theory and application of atomic absorption.

2. Goodfellow, G. I.; Simple Interchangeable Hollow-Cathode Lamps for Use in Atomic-Absorption Spectrometry. Applied Spectroscopy, Vol. 21, 1967, pp. 39 - 42.

   Describes the design of demountable hollow-cathode lamps into which the sample may be introduced and ionized in an electrical discharge. Lamps are operated in a reduced atmosphere of argon.

3. Polucktov, N. S.; Technics in Flame Photometric Analysis, Van Nostrand, Princeton, N. J., 1966.

   A book which reviews methods of analysis by flame photometry.

4. Pungar, E.; Flame Photometry Theory, Van Nostrand, Princeton, N. J., 1967.

   A book which reviews the theory of analysis by flame photometry.

5. Rechnitz, G. A.; Ion-Selective Electrodes, Chem. and Eng. News, Vol. 45, June 12, 1967, pp. 146 - 158.

   A very recent review of advances in the development of ion-selective electrodes. Three types of electrodes are discussed: glass, solid-state or precipitate, and liquid-liquid membrane. Ion-selective electrodes, which measure activity rather than concentration, have been developed for such difficult ions as sodium, potassium, calcium, ammonium, magnesium, and fluoride.

3.3.31 Sulfates

A. Principles of present methods

1. Method 1, Kleeman et al.[3] modified by Henry[2]

    Serum is deproteinized with uranyl acetate. Addition of benzidine to the filtrate precipitates the sulfate as benzidine sulfate. The benzidine in the precipitate (benzidine sulfate) is quantitated colorimetrically by reaction with sodium β-naphthoquinone-4-sulfonate.

    Procedure: Benzidine is added to the protein-free filtrate of serum and the mixture is allowed to stand at 4° C for 3 hours. The benzidine sulfate is separated by centrifugation and is washed with alcohol-ether. The benzidine sulfate is dissolved in borate buffer with heat at 60° C. This addition of naphthoquinone reagent results in the formation of a colored complex the absorbance of which is measured at 485 mμ. The method is sensitive and has a good precision. However, it requires the use of volatile reagents (alcohol, ether, acetone) and the naphthoquinone reagent is very unstable.

    Uranyl acetate has been selected as the protein precipitating reagent because it also precipitates the interfering phosphates. The method is applicable to urine.

2. Method 2, Miller et. al.[4]

    A protein free, phosphate-free filtrate is obtained by mixing plasma with a solution of uranyl acetate. A standard $Ba^{133}Cl_2$ solution is added to the filtrate resulting in $BaSO_4$ precipitation, which is allowed to proceed for 4 hours at 5° C.

    The mixture is centrifuged and an aliquot of the supernatant is removed and counted in a scintillation well counter. The concentration of sulfate is calculated by the equation

    $$M_s = \frac{CoVb - C(Vs + Vb)}{CoVb} \times \frac{(Vp + Vu)Vb}{VsVp} \times Mb,$$

    where

Co = counting rate of the standard $Ba^{133}Cl_2$ solution.

C = counting rate of the Ba^{133} in the supernatant solution.

Vp = volume of plasma

Vu = volume of uranyl acetate solution

Vs = volume of supernatant counted

Vb = volume of barium solution added

Mb = concentration of $Ba^{133}Cl$ solution

Ms = concentration of sulfate in plasma

If Vp = 1, Vu = 2, Vs = 1, and Vb = 0.5, the equation becomes

$$Ms = 1.5 \times \frac{Co - 3C}{Co} \quad Mb.$$

This method requires less handling than Method 1 and the use of volatile reagents is avoided. The authors claim that their results are in good agreement with those of Kleeman et al.[3] The specific activity of the $BaCl_2$ used was 0.12 mc per gram of Ba.

3. Method 3, Berglund and Sorbo[1]

Serum is deproteinized with trichloroacetic acid. To the clear filtrate a $BaCl_2$ - gelatin reagent is added which precipitates the sulfate as $BaSO_4$. The absorbance of the turbid solution is measured at 360 mμ and compared to that of a standard sulfate solution. The relationship between sulfate concentration and absorbance is linear. The same procedure can be employed for the measurement of inorganic sulfate in urine. The specificity and precision of this method need to be evaluated.

B. Applicability of present methods to space flight conditions

1. Merit table

Sulfates

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	Fair
2. Sample size	1 ml	1 ml	1 ml
3. Time required	5 hours	5 hours	1 hour

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Sulfates - continued

Merit Parameters	Method 1	Method 2	Method 3
4. Reproducibility	8	8	-
5. Suitability for null gravity use	2	7	7
6. Overall safety	5	5	6
7. Nontoxic reagents	4	9	8
8. Noncaustic reagents	9	9	5
9. Overall safety	5	5	6
10. Insensitive to environmental changes	2	9	3
11. Ease in training personnel	1	2	7
12. Degree of separation required (10=none)	2	6	8
13. Minimal handling by analyst	1	4	6
14. Common use of analytic equipment	8	2	8
15. Nondestructive of sample	1	1	1
16. Merit range	-	-	0-46
17. Mean figure of merit	17	36	20

2. Discussion

Serum: None of the methods can be recommended at the present time. However the method of Berglund is relatively simple and it could be applicable if its reliability, sensitivity, and precision are established.

Urine: Method 3 is recommended. The sensitivity of the method is more than adequate.

Equipment

Method 1: Centrifuge, spectrophotometer, heating bath, refrigerator

Method 2: Scintillation well counter, centrifuge, refrigerator

Method 3: Spectrophotometer, centrifuge

C. Areas for research and development

Certain anaerobic bacteria can utilize sulfate as their biologic oxidant. It may thus be possible to employ such microorganisms in as-

saying sulfate content.

D. References

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   Addition of $BaCl_2$ - gelatin reagent to a protein free filtrate results in the precipitation of $BaSO_4$ which is measured by turbidimetry.

2. Henry, R. J.; Determination of Sulfate in Clinical Chemistry, Principles and Technics, Hueber Med. Division, Harper and Row Publishers, 1964, pp. 417 - 421.

   A book devoted entirely to the principles and techniques of chemistry. The author generally discusses several methods in some detail and then selects one or two and describes them in great detail. Provides values of accuracy, precision and normal values for selected procedures.

3. Kleeman, C.R.; Taborsky, E.; Epstein, F.H.; Improved Method for the Determination of Inorganic Sulfate in Biologic Fluids, Proc. Soc. Exp. Biol. Med., Vol. 91, 1956, p. 480.

   Describes a method for the estimation of serum inorganic sulfate using benzidine and naphthoquinone sulfonate.

4. Miller, E. et al.; The Use of Radioisotopes to Measure Body Fluid Constituents 1. Plasma Sulfate, J. Lab. Clin. Med., Vol. 58, 1961, pp. 656 - 661.

   Describes the use of Ba^{133} for the determination of inorganic sulfate.

3.3.32 Zinc in blood, serum, or urine

The determination of zinc in biological fluids presents serious difficulties. Zinc can be determined colorimetrically with dithizone, by neutron activation analysis, emission spectrometry, atomic absorption spectrophotometry and x-ray fluorescence.

A. Principles of present methodology

    1. Method 1, Burns et al.[1]

Principle: The method depends upon the formation of radioactive zinc by bombardment of the sample with a flux of neutrons. The radioactive zinc is then measured by a sensitive device.

Procedure: Blood is collected in quartz ampoules and first is dried at 70° C and then at 110° C for a total of 13 hours. The ampoules are sealed and irradiated in a thermal neutron flux of 2×10^{12} to 2×10^{13} $n/cm^2/sec$ for 3 days. After a decay interval of 2 days the organic material is digested with H_2SO_4-H_2O_2. The various elements present in the ash are separated by distillation upon addition of hydrobromic acid (HBr), passage through ion-exchangers and selective elution. The gamma-spectrometric measurements are made with a 512-channel pulse height analyzer.

    2. Method 2, Johnson[3]

Principle: Zinc analysis is made on a deproteinized sample at pH 9.5 by titrating with tetraethylenepentamine (Tetren) potentiometrically, using dual, gold, polarized electrodes to the respective end-point. The latter is determined from the inflection point on a graphic plot or where the potential is at a minimum.

Procedure: Serum is deproteinized with 1% picric acid solution. Picric acid is removed from the protein free filtrate by absorption on a Dowex column. The zinc ions are eluted from the column by elution with deionized water. The pH of the eluate is adjusted to 9.5 with NH_3-NH_4NO_3 buffer and NaCN and the zinc is titrated with

a solution of Tetren. The author claims very good precision for this method. The results obtained are in good agreement with colorimetric methods. The presence of sulfates and halide ions is undesirable.

3. Method 3, Helwig, et al.[3]

Principle: Serum or urine are digested with HNO_3 -H_2SO_4 -$HClO_4$ mixture. The acid-digest is neutralized with ammonia and then the pH is adjusted to 5.7, with thiosulfate-cyanide-acetate buffer. The zinc is extracted from the solution with a carbon tetrachloride solution of dithiocarbazone (dithizone). The absorbance of the zinc dithizonate is measured at 525 mμ in a spectrophotometer. If urine is analyzed a volume of 5 ml is required.

B. Suitability of present methodology to space flight conditions

1. Merit table

Zinc

Merit Parameters	Method 1	Method 2	Method 3
1. Sensitivity	Good	Good	Good
2. Sample size	2 ml	2 ml	2 ml
3. Time required	10 days	15 min	3 hours
4. Reproducibility	7	9	8
5. Suitability for null gravity use	1	1	1
6. Overall safety	0	3	1
7. Nontoxic reagents	0	1	1
8. Noncaustic reagents	0	7	0
9. Specificity	9	9	7
10. Insensitive to environmental changes	-	-	-
11. Ease in training personnel	0	2	2
12. Degree of separation required (10=none)	0	4	5
13. Minimal handling by analyst	0	3	1
14. Common use of analytic equipment	0	1	9
15. Nondestructive of sample	0	0	0

Merit Parameters	Zinc Method 1	Method 2	Method 3
16. Merit range	3-5	12-16	6-8
17. Mean figure of merit	4	14	7

2. Discussion

Choice of method: Method 2 if modified extensively could be applicable. The deproteinization step may be altered by absorption of the serum proteins on a Sephadex column. The burette can be replaced by a syringe-type burette and titration can be performed in a closed system.

Equipment

Method 1: Heater, neutron flux, ion-exchange columns, 512-channel pulse height analyzer, quartz ampoules

Method 2: pH-meter, magnetic stirrer, centrifuge, mercury electrode, chromatography columns, burette

Method 3: Heater, spectrophotometer

C. Areas for research and development

The titration method is attractive and should be thoroughly investigated.

Atomic Absorption Spectrophotometry is quick and specific but the flame requirement makes the method unacceptable.

D. References

1. Brune, D.; Samsahl, K.; Wester, P.O.; A Comparison Between the Amounts of As, Au, Br, Cu, Fe, Mo, Se and Zn in Normal and Uraemic Human Blood by Means of Neutron Activation Analysis, Clin. Chim. Acta., Vol. 13, 1966, pp. 285 - 291.

Zinc and other elements were measured in blood by gamma-spectrometric analysis after blood is dried, irradiate, digested, etc.

2. Helwig, H.L., et. al.; Modified Zinc Analysis Method and Serum and Urinary Zinc Levels in Control Subjects, Am. J. Clin. Path., Vol. 45, 1966, p. 45.

Zinc is measured colorimetrically with dithizone.

3. 4   NEW ANALYTICAL METHODS

3. 4. 1   Introduction

The analytical methods discussed in Section 3. 3 present essentially the current state of the art.   These methods have not necessarily been proven for all the analyses studied during this program, but the technology is established and the problems remaining involve primarily engineering to achieve compatibility with the space environment.   The major engineering effort would have to be devoted   to the designing of (1) a suitable spectrophotometer for use in the ultraviolet, visible, and near infrared portions of the spectrum, (2) a centrifuge for separating serum and plasma from whole blood and electrophoretic or chromatographic apparatus for protein separation or removal, and (3) apparatus for transfer of samples and reagents from one closed system to another without endangering the crew.   Given suitable instrumentation in these three areas, it seems entirely reasonable that all the practical methods discussed so far could be implemented within a period of one to three years.   This period includes the launching of the Apollo Applications Program and the establishment of an orbiting research laboratory.

Admittedly the methods of Section 3. 3 do not greatly advance the state of the art in clinical analysis, which in some areas has not changed significantly in two decades.   There are a number of reasons for this lag in clinical technology, but perhaps the most important has been the inability of medical research centers to obtain support for research which would ultimately advance the state of clinical technology.   It is through promotion of research, particularly the development of new applications, methods, and analytical techniques, that private foundations and government agencies such as NASA can contribute significantly to advancing clinical technology.   Such efforts could provide direct support for the space program while simultaneously easing the developing crisis in clinical laboratories.   A good example of an analytical technique that is currently showing significant advances is the ion-selective electrode.[*]   Development of new glasses permeable to specific ions has made possible a whole family of electrodes capable of identifying ions in solution, many of which have heretofore

[*] See reference 5 under section 3. 3. 30

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been difficult to detect. Since such electrodes measure ionic activity rather than concentration, considerable work remains to be done in correlating activity levels with physiological conditions before ion-selective electrodes can be adopted for routine clinical analyses. Given sufficient support, these problems could probably be resolved within five years.

Three approaches to the analysis of certain biochemical constituents show promise of becoming simple, effective methods, but they will require considerable research to determine the full range of their applicability. They are neutron activation analysis, microcalorimetry, and the analysis of optical spectra. Neutron activation analysis has been studied extensively and employed in the detection of many elements. Biomedical applications of this technique, however, have not yet received the attention given to other applications of activation analysis. The radiation background of the space environment effectively limits the number of elements that could be detected in a practical orbiting laboratory. Within a period of a year or two this technique could probably be applied to the analysis of seven or eight of the constituents included in this study. A somewhat longer period might be required to develop microcalorimetric techniques, but they offer the possibility of analyzing a single small specimen for a large number of proteins and antibodies with one instrument. Very little work has been done so far in applying microcalorimetry to biomedical analyses. The analyses of optical spectra and their dispersion will require much more work but may eventually become a powerful analytical tool. A period of five to ten years will be necessary to establish the feasibility of this approach and the range of its applicability. These three approaches will be discussed in the sections to follow.

3.4.2 Neutron Activation Analysis

Neutron activation analysis provides a convenient, precise method for determining the concentrations of a number of elements present in samples undergoing analysis. In most cases it has the advantage of requiring no sample preparation other than encapsulation, permitting the samples to be handled

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throughout the analysis in closed containers.

Neutron activation consists of irradiating the samples with neutrons and observing the residual activity to determine the quantities of various elements present. It is potentially applicable to sodium, potassium, chlorine, calcium, magnesium, manganese, phosphorus and zinc in this study.[1] It should be emphasized, however, that only the quantity of an element is measured; for example, no distinction would be made between chloride, chlorate, or any other valence state of chlorine.

Sealed polonium-beryllium neutron sources of sufficient neutron yield for activation of small biochemical samples, such as those being considered here, are commercially available.

Possible problem areas are the weight of the electronic equipment needed for radiation detection and the weight of radiation shielding which might be needed around the neutron source for the radiological health safety of the crew and around the detector to limit the background count rate to an acceptable level. The background radiation appears to be a serious limitation on the feasibility of using this method in any but low earth or synchronous orbits. Shielding can be increased only to a certain thickness before secondary emission begins to nullify the effect of the shielding.

Should the weight of the circuitry and shielding prove to be prohibitive for the single purpose of biochemical analyses, additional experiments which might be carried out with this equipment should be considered for possible justification for orbiting the equipment. The detection equipment conventionally used in activation analysis is highly versatile, and with little additional equipment might provide the basis for several other worthwhile experiments.

Reference

1. Spencer, R. P.; Mitchell, T. G.; King, E. R.; Medical Applications of Neutron Activation Analysis, Int. J. Appl. Rad. Vol. 3, 1958, pp. 104 - 112.

Exposure of 10 ml serum to a flux of 2.5×10^8 neutrons/cm^2/sec for one hour produced 15 radioisotopes of 12 elements to the extent of 30 dpm or greater: Na, K, Ca, Cu, Zn, Mg, Co, Mn, Cl, Br, I, P.

3.4.3 Microcalorimetry

The use of specific enzymes or other specific binding proteins to analyze constituents of blood, urine, feces, or sweat is highly desirable, because such reagents permit analysis with a minimum of separation of constituents. The techniques of microcalorimetry offer a means of quantitating individual constituents by measuring the heat of each enzymatic reaction. Although the principles of classical microcalorimetry are well known, practical applications to biomedical analyses have only recently begun to receive attention, following the development of a new method of microcalorimetry[3] and the introduction[1] in 1965 of a commercial calorimeter* capable of accurately measuring total heats of 5 milli-calories and 10 microcalories per second of continuous heat flow by the heat-burst principle.

Theoretically one should be able to analyze a single sample of body fluid for a number of constituents by successively adding specific reagents and measuring the heat of reaction. The reagents, a number of which are commercially available, can be lyophilized on earth and reconstituted as needed in space. The technique of microcalorimetry might well permit analyses of constituents with similar spectral characteristics. It might also make possible the detection of specific binding of molecules when precipitation does not occur, as in the case of nonprecipitating antigen-antibody complexes.[14]

The following discussion will illustrate how microcalorimetric techniques might be developed to analyze selected constituents of interest to this study. Specific enzymes[10] are presently known which might be applied to microcalorimetric determinations of creatine, creatinine, pyrophosphate, blood urea nitrogen, glucose, amino nitrogen, and lactic acid. Creatine phosphotransferase catalyzes the transfer of the phosphate radical from adenosinetriphosphate (ATP) to creatine.[12] With an excess of ATP and the enzyme, the reaction should go to completion with the production of adenosinediphosphate and phosphocreatine. Pyrophosphate[2,5] might be determined by reaction with pyrophosphatase and measurement of the heat of hydrolysis. Similarly, the determination of urea would employ the enzyme urease.[6] Glucose oxidase[7,12] could be used for determinations of glu-

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* Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.

cose, and L-amino acid oxidase[8] could be used for analyses of amino nitrogen. Lactic acid could be determined by adding a cofactor, lactic acid dehydrogenase[9] and blue tetrazolium dye and monitoring the oxidation of lactate. Quantitative determinations of the enzyme alkaline phosphatase[4,11] would involve the addition of a suitable substrate and microcalorimetric measurement of the reaction rate rather than the total heat of reaction. Microcalorimetric analyses for particular proteins and antibodies[14] would be based on reactions with appropriate antibodies or antigens.

Many metal ions are known to act as specific activators of certain enzyme systems. This property may be utilized for their measurement by studying the rates of enzymatic reactions as a function of the concentration of various metallic ions.

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    General description of a new method of microcalorimetry and a suitable commercial microcalorimeter.

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    Describes calorimetric measurement of hydrolytic reaction.

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    A new method of microcalorimetry (heatburst microcalorimetry), which measures heat of reaction by rapid dissipation and recording of heat flow rates with time, is described.

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    Describes assay and properties of alkaline phosphatase.

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Description of microcalorimetric techniques as applied to antigen-antibody reactions.

3. 4. 4 Analysis of Optical Spectra

Technological advances in recent years are making possible the application of optical analysis techniques to problems never before amenable to solution in this fashion. The principles of optical spectroscopy have long been known and recognized as providing detailed information about substances on the atomic and molecular levels. The term "optical spectroscopy" as used here refers to that portion of the electromagnetic spectrum ranging from the ultraviolet through the visible and into the infrared. It includes such specializations as absorption spectroscopy (visible and infrared), emission spectroscopy, fluorophotometry, and phosphorimetry, infrared spectroscopy, optical activity and rotatory dispersion, and Raman spectroscopy.[1] Most substances exhibit a spectral dispersion in these physical characteristics, and in the uniqueness of such spectra resides the power of these techniques. However, it is this multiplicity of spectra, often superimposed on one another, as in mixtures, which has so far prevented the full exploitation of optical techniques. Only with the advent of high speed computers has it become possible to approach the problem of resolving, identifying, and quantitating complex spectra on a practical level. Indicative of the progress being made in this area has been the introduction within the past two years of analog computers capable of resolving the complex spectra produced by such techniques as chromatography, electrophoresis, spectroscopy, ultracentrifugation, and x-ray diffraction.[2] Utilization of such computers would be an absolute necessity in any program for analysis of optical spectra. The use of computers would also make possible the intercomparison of data from multiple measurements using a variety of optical techniques as well as other methods of physical analysis.

The types of optical spectroscopy which are most readily adaptable to the space environment and which will be discussed in the paragraphs to follow are fluorescence and phosphorescence spectroscopy, optical activity and rota-

tory dispersion, and absorption spectroscopy.

Fluorescence spectroscopy is based on the ability of certain atoms and molecules to emit radiation at specific wavelengths during electronic transitions from higher to lower energy states. The atom or molecule may be excited by electromagnetic radiation, by mechanical means, by chemical means, by electron bombardment, and in numerous other ways. Fluorescence may be a natural characteristic of a substance or it may be induced by chemically binding a chromophore to the substance. Analytical parameters of fluorescence spectroscopy include indentification of the excitation source and identification and quantitation of the fluorescent lines and bands.

Phosphorescence spectroscopy differs from fluorescence spectroscopy primarily in the time lapse between excitation and emission. Fluorescent radiation is restricted to emissions occurring between 10^{-8} and 10^{-3} seconds to several hours after excitation. This time lapse introduces an additional parameter which can aid in the identification of biochemical constituents.

Optical activity and rotatory dispersion (ORD) refer to the rotation of the plane of polarization in optically active substances and the spectral variation of this rotation. Many substances of interest to clinical biochemistry exhibit intrinsic optical activity, and optical activity can be induced in other substances by a variety of physical and chemical means. Spectropolarimetric analyses of biochemical mixtures involve the resolution of ORD curves into spectra of individual constituents and the quantitation of each substance in the sample.

Absorption spectroscopy, one of the most frequently employed methods of optical analysis, finds application in the ultraviolet, visible, and infrared portions of the spectrum although most clinical applications at present are restricted to the frequencies of visible light. A major problem retarding the growth of absorption spectroscopy is the analysis of polycomponent systems. The analysis of single component solutions is well established, although much work needs to be done on increasing the reproducibility of spectrophotometers (to the point where the absorbance index becomes a reliable characteristic) and in broadening the spectral range of accurate analysis (so that the accuracy of infrared analyses approaches

the 0.1 percent accuracy[3] attainable in visible analyses). The analysis of poly-component systems has developed slowly and is largely restricted to situations in which the absorbance curves of the constituents do not overlap in the region of measurement or, if overlap does occur, correction can be made for the interference. A third situation arises in which simultaneous equations for the absorbance of each constituent must be solved. High speed computers should find increasing application to this problem.

The use of optical analyses in biomedical studies in space offers a number of potential advantages, as indicated in the following paragraphs. A concentrated research effort, however, will be required to realize the full possibilities in practice.

A. Sensitivity

Ultra high sensitivity is often not a requirement for biochemical analyses, but it is requisite for determinations of trace constituents. Luminescence spectroscopy has already established itself as one of the most sensitive analytical tools available. Present technology permits the detection, for example, of lucigenin[5] in aqueous solutions in dilutions as high as $1:10^{10}$; yet even this sensitivity can be expected to improve with further research.

B. Preparation

The analysis of optical spectra often permits identification and quantitation of biochemical constituents without the need of prior chemical preparation, as in the recording of optical absorption spectra, in atomic absorption spectroscopy, and in some cases, emission spectroscopy. The further development of luminescence spectroscopy and spectropolarimetry offer the possibility of analyzing ever more complicated mixtures without extensive preparation. Theoretically, space environment offers an exceptional opportunity to lyophilize samples by venting to space. The removal of water by lyophilization will simplify optical measurements by removing the solvent. In particular, absorption measurements in the infrared will benefit from the elimina-

tion of the water absorption bands which frequently mask other absorption bands. Techniques exist currently for compressing powdered material into thin micropellets for absorption measurements. An additional technique which bears investigation is the use of thin film smears on quartz plates (or other suitable material) for optical measurements over a wide spectral range.

C. Specificity

It is in this area that the greatest amount of research is needed to establish the degree of specificity attainable by optical methods. Because of the large number of modes of excitation available to organic molecules, it is theoretically possible for many substances to exhibit unique fluorescence, phosphorescence, and optical rotatory dispersion spectra. The use of specific binding chromophores, such as the acridine dyes,[7] will increase the number of constituents amenable to analysis by optical methods. The use of high speed computers will be requisite to resolve, identify, and quantitate the complex spectra that can be expected in optical analyses.

D. Sample Size

Biochemical analyses in space will generally require the utilization of small samples, especially in the case of blood analyses. Optical analyses offer a distinct advantage, particularly in luminescence spectroscopy and in spectropolarimetry where a microscope can be used to significantly reduce the sample volume necessary for analysis.

E. Automation

The practical requirement of a computer for analyses of optical spectra makes the possibility of nearly complete automation of analyses a reasonable expectation. (The term "automation" as used here, refers not to the automated processing of multiple samples, but rather to the computer control of sequential operations such as operating a spectrophotometer, recording a spectrum, analyzing the spectrum to identify and quantitate each constituent in the sample, and recording

or transmitting the completed analysis.) This is an area where practical spin-offs could benefit terrestrial laboratories, especially clinical laboratories, where the addition of automatic multiple processing would substantially accelerate operation.

F. Nondestructive Analysis

To the extent that optical analyses can be implemented without chemical preparation, such analyses will be nondestructive and the same sample can be subjected to analysis by other methods, either as redundant checks on the optical analyses or for determination of other constituents.

G. Safety

A primary advantage of physical methods of analysis is the potential safety afforded to the crew of the spacecraft. If chemical preparation of the sample can be obviated, a prime source of possible contamination of the spacecraft or its atmosphere can be eliminated. Most optical methods lend themselves readily to the analysis of sealed samples, giving added protection to the astronauts.

Other potential advantages could undoubtedly be unumerated, but the brief discussion above will indicate some practical goals for research directed toward the further development of optical methods.

Instrumentation for the study of fluorescence spectroscopy and spectorpolarimetry is currently being developed. A spectropolarimeter designed for the detection of extraterrestrial life[6] is well suited to the problem of biochemical analyses in an orbiting laboratory inasmuch as it is insensitive to depolarization scattering, it does not require intense light sources, and it is economical of power. It is designed to operate in the presence of optical densities of five or more, permitting the use of almost any chromophore which might be needed, and its useful spectral range extends into the ultraviolet, where the Cotton effect is generally most pronounced. Analyses of optical spectra represent an approach to biochemical analysis which appears to be well suited for

utilization in the space environment. The details of analytical methods for specific substances, the full extent to which the potential advantages discussed above can be realized, and the design of suitable instrumentation will require extensive research. The research required is not so much basic research, although this will be needed to establish the various spectra and other optical properties of individual substances, but rather research directed towards specific applications and the development of practical techniques for accurately analyzing mixtures of constituents such as blood, urine, and sweat.

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(Annotation not available.)

Section 4

RECOMMENDATIONS FOR FUTURE WORK

This study has revealed that physical methods have not found extensive use in biochemical analyses but that, in principle, physical methods should be applicable to the analyses of some constituents of blood, urine, sweat, and feces. This report has sought to indicate which analytical procedures are adaptable to the inclusion of physical methods of analysis and how such adaptations might be effected; furthermore, it has sought to survey selected physical methods of analysis which have not been fully developed or extensively applied to biochemical analyses. A realization of the desirability of physical methods for biochemican analyses in space and of the present state of development of such methods has led to the drafting of the following recommendations for future investigation.

1. The new analytical methods of Section 3.4 will require extensive investigation before working procedures can be developed. These investigations can be divided into at least four phases: the study of single purified constituents, the study of simple mixtures containing perhaps two or three constituents, the study of complex mixtures such as body fluids, and the design of flight instrumentation.

    A. Single Constituents

    A considerable body of literature exists on the properties of individual substances, and full advantage should be taken of this information already available. Where individual substances of interest have not been adequately studied, directed programs must be undertaken to determine those properties requisite to analytical determinations. Such studies should provide knowledge of various spectra and their dependence on environmental conditions and concentration levels.

    B. Simple Mixture

    A major goal of the study of simple mixtures must be the determination of conditions when spectra are linearly additive and

when the mixing of constituents may alter the spectra of the individual constituents. Experience must also be gained in resolving composite spectra into spectra of individual constituents and interpreting these spectra in terms of concentration levels. In the case of microcalorimetric determinations this phase of investigation might consider the accuracy with which a particular heat of reaction can be measured (and interpreted) in the presence of other constituents.

C. Complex Mixtures

The logical step following a successful investigation of simple mixtures would be a study of more complicated mixtures, culminating in investigations of actual body fluids. It may be found for instance, that an effective limit on the number of constituents that can be resolved will require some prior separation of body fluids.

D. Design of Instrumentation

The final phase of developing analytical procedures for an orbiting laboratory will be the design, manufacture, and testing of instrumentation compatible with the environment and the analytical requirements.

2. For immediate or near future analyses, reliance must be placed on current methods or adaptations of current methods. It will usually be necessary to modify analytical procedures or equipment in order to make them compatible with the space environment. In cases where a recommended procedure involves the combining of accepted methods or some other adaptation of a method, it will be necessary to verify experimentally the effectiveness of the proposed procedure before attempting to adapt the method to space use.